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(54) Title: ANTI-NEUTROPHIL CYTOPLASMIC ANTIBODY MATERIAL ASSOCIATED WITH ULCERATIVE COLITIS AND RELATED METHODS AND KITS

(57) Abstract

For the first time, the present invention provides isolated, substantially pure, and/or recombinantly produced pANCA associated with ulcerative colitis (UCpANCA and UCpANCA material) as well as polynucleotides encoding the UCpANCA, UCpANCA material and UCpANCA polypeptides of the present invention. Using the phage display technique, immunoglobulin gene repertoires biased for the immunoglobulin genes utilized by pANCA seropositive UC patients have been isolated, amplified, randomly re-combined to generate phagemid expression vector libraries encoding DNA homologs of the gene repertoire. Methods are provided for enriching these libraries to create libraries of V_H- and V_L-encoding DNA homologs having immunoreactivity of UCpANCA antigen. Also provided are methods of screening the libraries of the present invention for UCpANCA and UCpANCA material. These libraries may be encapsulated in phage particles or in cells. Methods and kits are also provided for screening for UCpANCA in a sample and isolating UCpANCA antigen.

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ANTI-NEUTROPHIL CYTOPLASMIC ANTIBODY MATERIAL ASSOCIATED WITH ULCERATIVE COLITIS AND RELATED METHODS AND KITS

I. ACKNOWLEDGMENT

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II. BACKGROUND OF THE INVENTION

A. The Antibody Repertoire

Over a lifetime, a person confronts the possibility of infection with an almost infinite number of unique foreign substances (antigens). Since it could never be anticipated which of these antigens will ultimately infect a person, it is beneficial that the body possesses an elegant system of producing an equally infinite array of antibodies which recognize, bind and trigger the destruction of antigens.

Antibodies are Y-shaped, tetrameric molecules consisting of a pair of identical, relatively long polypeptide chains called heavy (H) chains and a pair of identical, shorter polypeptide chains called light (L) chains. Each arm of the Y shaped structure is comprised of one light chain and one end of a heavy chain bound together by a single disulfide bond. At the juncture of the arms, the two heavy chains are bound to each other by two disulfide bonds to form the stem of the Y shaped structure.

This architectural description of an antibody, although visually appealing, can be deceptively simplistic. Antibody architecture accommodates a wealth of structural diversity. Both the heavy and light chains contain variable (V) and constant domain. These V domains are responsible for antigen binding.

Heavy and light chain variable domains each consist of B-sheet scaffold, surmounted by three antigen-binding loops (complementarity-determining regions or CDRs) of different lengths which are fleshed with a variety of side chains. The CDRs are the most diverse regions of the antibody molecule; all six associate to one degree or another in forming the site at which the antibody binds its antigen (antigen-binding site). The structural diversity of the loops can create binding sites of a variety of shapes, ranging from almost flat surfaces to deep cavities.

Thus the vast array of antibody specificities depends on the diversity of the variable domain structure which in turn depends on the diversity of the primary sequence of the V domain. Underpinning the structural diversity of antibodies is a combinatorial genetic diversity. Heavy and light chain polypeptides are each encoded by an ensemble of gene segments selected from immunoglobulin (Ig) gene complexes. During the maturation of B-cell (the cells which produce antibodies), discontinuous gene segments within these gene complexes undergo a series of somatic rearrangements to form the nucleic acid sequence that ultimately may encode the heavy and light chains of the antibody molecule.

Generally in humans, the first Ig gene rearrangements occur within the Ig heavy chain gene complex. The variable heavy chain domain is generated by the assembly of a V_HDJ_H exon from three separate germline DNA segments. One or more diversity (D) gene segments (selected from more than two dozen D germline gene segments) is joined with a single joining (J_H) gene segment (selected from about six functional J_H germline gene segments). The resulting DJ_H complex may then rearrange with a V_H gene segment to form a V_HDJ_H exon that may encode the variable portion of the antibody heavy chain. About 120 germline V_H gene segments

(of which only about 80 are potentially functional) are available for Ig gene rearrangement and can be divided into at least six families in the basis of nucleotide homology of 80% or above.

After successful V_HDJ_H rearrangement, a similar rearrangement occurs to produce the light chain. One of approximately 70 kappa variable gene segments (V_K) rearrange to one of five J_K gene segments thereby generating an exon that may encode a kappa light chain variable domain. Should this rearrangement fail to generate a functional gene, then one of approximately 70 lambda light chain variable gene segments (V_λ) may rearrange to one of four functional J_λ-C_λ complexes to generate an exon that may encode a lambda light chain variable domain. The final products of such genetic gymnastics are the somatically generated genes that encode the two polypeptide chains of the antibody molecule.

Two of the heavy chain CDRs (1 and 2) are encoded by the V_H segment. The heavy chain CDR3 is the most variable portion of the antibody molecule and is encoded by the 3' end of the V_H gene segment, the D segment and the 5' end of the J_H segment. With nucleotide addition (N-region diversity at the V_H-D and D-J_H junctions) the use of different reading frames in the D segment, and the combination of different rearranged heavy and light chains, the diversity of primary antibody libraries is huge. During an immune response, the antibody variable domains are further diversified by somatic hypermutation, leading to higher affinity binding of the antigen.

B. Autoantibodies

The monumental repertoire of the adaptive immune system has evolved to allow it to recognize and ensnare virtually any shaped microbial molecule either at present in existence or yet to come. However, in doing so it has been unable to

avoid the generation of autoantibodies: antibodies that bind with the body's own constituents and trigger an immunological path of destruction.

Natural immunological tolerance mechanisms prevent the expanded production of autoantibodies. After antibody gene rearrangement, virgin B-cells (the cells that generate antibodies) that display autoantibodies are destroyed or suppressed by the bodies tolerance mechanisms. Despite this safety-net, autoantibodies are still produced and for many people create no recognizable pathogenic disorder. It has been estimated that 10-30% of B cells in normal, healthy individuals are engaged in making autoantibodies. Production of autoantibodies is not only the result of an exceptionally diverse immune system, an immune response against one's self but, can also arise in autoimmune disease or after infections.

C. **Inflammatory Bowel Disease**

Inflammatory bowel disease (IBD) is the collective term used to describe two gastrointestinal disorders: ulcerative colitis ("UC") and Crohn's disease ("CD"). Although the diseases have distinct pathophysiological characteristics, they are frequently considered together due to several clinical and therapeutic similarities. Excluded from this category, however, are gastrointestinal inflammatory disorders of known infectious, toxic or ischemic etiology which may mimic IBD acutely, but do not cause a chronic relapsing and remitting syndrome.

IBD occurs world-wide and is reported to afflict as many as two million people. The course and prognosis of IBD is widely variable. Onset has been documented at all ages; however, IBD predominately begins in young adulthood. The three most common presenting symptoms of IBD are diarrhea, abdominal pain, and fever. The diarrhea may range from mild

to severe and is often accompanied by urgency and frequency. In UC, the diarrhea is usually bloody and may contain mucus and purulent matter as well. Anemia and weight loss are additional common signs of IBD. Ten percent to fifteen percent of all patients with IBD will require surgery over a 10-year period. The risk for the development of cancer is increased in patients with IBD as well, particularly in those with UC. The longer the duration of disease, the higher the risk of developing carcinoma. Patients with UC regularly undergo cancer surveillance by endoscopy after ten years of disease. Reports of an increasing occurrence of psychological problems, including anxiety and depression, are perhaps not surprising secondary effects of what is often a debilitating disease that occurs in people in the prime of life.

D. Methods of Diagnosing IBD

Inflammatory bowel disease poses a clinical and scientific challenge to physicians and researchers. To date most of the diagnostic tools for IBD are quite subjective. A battery of expensive and invasive laboratory, radiological, and endoscopic evaluations are combined to derive a diagnosis of IBD and to assess the extent and severity of the disease. Nevertheless, differentiating UC from CD, as well as other types of inflammatory conditions of the bowel, such as irritable bowel syndrome, infectious diarrhea, rectal bleeding, radiation colitis, and the like, is difficult, because the mucosa of the small and large intestines reacts in a similar way to a large number of different insults. Consequently, the initial symptoms are often confused for non-chronic bowel disorders by physicians unfamiliar with IBD. As a result, IBD often goes mistreated and undiagnosed until the disease shows its chronicity which results in referral of the patient to a specialist. Even

then, the imprecise and subjective nature of endoscopic and radiologic examination can result in a misdiagnosis or indeterminate diagnosis even when the IBD is suspected. Unfortunately, the patient must often suffer as the disease progresses before a definitive diagnosis can be made. In many patients, though, the diagnosis of IBD must still be regarded as indeterminate because of the overlapping features of UC and CD, particularly with CD of the colon.

E. The Cause(s) of IBD are Unknown

Although the etiology of IBD is unknown, a number of studies have suggested that genetics is important in a person's susceptibility to IBD and that the immune system is responsible for mediating the tissue damage in these diseases. Generally speaking, a failure to down regulate the normal self-limited inflammatory response of the bowel is characteristic of IBD, but it remains unclear what initiates the pathogenic processes.

It has also been suggested that a primary abnormality of the immune system and its regulation might serve as primary initiating factors, or that the disease process might be initiated by an infectious agent and the injury is then perpetuated through immune-mediated or other processes. Although the mucosal injury observed during episodes of acute disease can resemble the effects of any of a number of recognized infectious agents, such as for example, *Campylobacter jejuni*, no transmissible infectious agent has been consistently identified with IBD.

Autoimmunity has been suggested in the pathogenesis of IBD. Evidence to suggest this hypothesis is based on the existence of circulating antibodies that react with unknown alimentary tract antigens of both human and animal origin. For example, human fetal and adult colonic, biliary, skin and vascular epithelial cells, epithelial cell associated

components from murine small intestine, rat and human colonic epithelial glycoproteins, intestinal bacterial polysaccharide, and antigens from germ-free rat feces have been reported to react with sera from patients with IBD. Other studies demonstrated an increased local IgG response in the colonic mucosa of patients with IBD and other colonic inflammations. The mechanism of this IgG response, the specific local antigens involved, and the role of these antibodies are unknown. While a wide range of immunologic abnormalities have been reported in these disorders, none other than the detection of anti-neutrophil cytoplasmic antibody ("ANCA" or more specifically "pANCA" as described below) in UC patients appear to be sufficiently reliable to be of diagnostic value. Isolation and identification of these antibodies and corresponding antigens would provide a powerful tool for elucidating the pathogenesis of UC and CD, ultimately leading to more effective treatment therapies.

F. Anti-Neutrophil Cytoplasmic Antibodies

Patients with certain chronic inflammatory conditions have been found to have serum antibodies to cytoplasmic components of the neutrophil ("ANCA" or "anti-neutrophil cytoplasmic antibody"). ANCAs have been divided into two broad categories based on the staining pattern generated by immunofluorescent microscopy of alcohol-fixed neutrophils: cytoplasmic neutrophil staining ("cANCA") and cytoplasmic staining with perinuclear highlighting ("pANCA"). Unfortunately, these staining patterns do not always accurately reflect the cellular localization of the reactive antigens. Generally, the literature has considered this perinuclear staining pattern to be an artifact of alcohol fixation which causes cytoplasmic granules to redistribute around the nucleus of the cell. Thus, although perinuclear staining may appear to detect nuclear binding in

neutrophils, such antibodies have still generally been regarded as binding to antigen of cytoplasmic origin. Nevertheless, these staining patterns have served as a basis for reliably distinguish between types of ANCA.

Recent studies have demonstrated the presence of pANCA in the serum of patients with UC. Saxon, et al., J. Allergy Clin. Immunol. 86:202-209 (1990). This pANCA identified in UC patients is unique from the cANCA associated with Wegener's granulomatosis and other systemic vasculitides both in its immunocytochemical staining pattern and in its antigenic target. In contrast to the perinuclear staining pattern exhibited by ANCA associated with UC, ANCA associated with Wegener's granulomatosis characteristically exhibits a granular, diffuse cytoplasmic immunofluorescence pattern. Duerr, et al., Gastroenterology, 100:1590 (1991). Furthermore, pANCA associated with UC can be differentiated from pANCAs occurring in non-UC patients by antigenic DNase sensitivity. By indirect immunofluorescence elevated pANCA levels are reported in 68-80% of UC patients and only very rarely in CD and other colitidies. Serum titers do not correlate with clinical status and high levels persist in patients even 5 years post-colectomy. Although pANCA is found only very rarely in healthy adults and children, healthy relatives of UC patients have an increased frequency of pANCA, suggesting that pANCA may be an immunogenetic susceptibility marker. Many putative antigens including lactoferrin, cathepsin G, and elastase have been proposed as the target antigen but investigators have demonstrated that these reactivities only account for a minor portion, if any of the pANCA activity associated with UC.

G. Isolation of pANCA associated with Ulcerative Colitis

Marker antibodies play a large role in diagnosing a diverse set of diseases, ranging from viral infections like HIV, to autoimmune disorders, such as lupus. These antibodies may directly cause pathology in which case a host of potential treatment modalities are implicated. Alternatively, these antibodies may be mere markers for the disease without directly causing tissue damage, or may aid in abrogating the infection as seen with most microbial infections. Therefore, whether they are responsible for the disease state or not, characterizing marker antibodies and their antigens can be very useful in both diagnosing a disease and in understanding immune disregulation that may underlie the pathogenesis.

Despite diligent efforts, attempts at isolating and identifying the structure of pANCA associated with UC have reportedly been unsuccessful. Several factors have made such identification difficult. Traditional methods of isolating antibodies by hybridoma production or EBV transformation are very time consuming and laborious. Unless the B cells producing the antibodies of interest are greatly over-represented, often these traditional methods fail simply because of the vast size and diversity of the native antibody repertoire. Even after the application of these conventional cloning strategies, pANCA associated with UC has reportedly remained unidentified after five years of intense study.

A surface-integration technology has been recently described for expressing a heterodimeric recombinant gene product such as an antibody molecule on the surface of a filamentous phage containing recombinant genes. The technology uses a filamentous-phage coat-protein as a membrane anchor for the recombinant gene product, thereby

linking gene and gene-product during the assembly stage of filamentous phage replication. This technique has proven useful in the cloning and expression of antibodies from combinatorial libraries. Kang et al., Proceedings of the National Academy of Science, USA, 88: 4363-4366 (1991); Barbas et al., Proceedings of the National Academy of Science, USA, 88: 7978-7982 (1991).) Using this technology, human combinatorial antibody libraries have been produced that immunoreact with hepatitis B virus surface antigens. Zebedee et al., Proceedings of the National Academy of Science, USA, 89: 3175-3179 (1992). The diversity of a filamentous phage-based combinatorial antibody library has been increased by shuffling of the heavy and light chain genes (Kang et al., Proceedings of the National Academy of Science, USA, 88: 11120-11123 (1991)), by altering the CDR3 regions of the cloned heavy chain genes of the library (Barbas et al., Proceedings of the National Academy of Science, USA, 89: 4457-4461 (1992)), and by introducing random mutations into the library by error-prone polymerase chain reactions ("PCR") (Gram et al., Proceedings of the National Academy of Science, USA, 89: 3576-3580 (1992)). In addition, single chain Fv fragments have been displayed on the surface of phage as described by Marks et al., Journal of Molecular Biology, 222: 581-597 (1991).

Despite recent developments such as these, there have been no reports of using these strategies to isolate pANCA associated with UC. Undoubtedly, this is attributable to some of the very same factors which have thwarted the attempt to isolate the antibody through the use of traditional hybridoma techniques, namely, failure to know the structure of the target antigen and failure to isolate a population of B-cells producing a sufficient amount of pANCA for meaningful study. Accordingly, the structural

identification and characterization of pANCA associated with UC has posed a daunting problem.

In view of the fact that diagnosing IBD is a prolonged, invasive and expensive process that often fails to resolve uncertainty until the diseases have shown their chronicity, and since IBD, and quite often its treatment, affects the lifestyle and functional capabilities of those afflicted, the need to identify and elucidate pANCA associated with UC is particularly poignant. The availability of pANCA would represent a major clinical advance which would aid in the diagnosis and therapeutic management of IBD and would provide the basis for design of more specific treatment modalities. In addition specific detection of UC in prospective parents can be useful in genetic counseling. Accordingly, there has existed a need for the isolation, identification and production of pANCA associated with UC for diagnostic, prognostic and therapeutic purposes.

III. BRIEF DESCRIPTION OF THE INVENTION

Patients with certain chronic inflammatory conditions have been found to have serum antibodies to cytoplasmic components of the neutrophil ("ANCA" or "anti-neutrophil cytoplasmic antibody"). ANCAs have been divided into two broad categories based on the staining pattern generated by indirect immunofluorescent microscopy of alcohol-fixed neutrophils: cytoplasmic neutrophil staining ("cANCA") and cytoplasmic staining with perinuclear highlighting ("pANCA"). Recent studies have demonstrated the presence of pANCA in the serum of patients with UC. This pANCA associated with UC can be differentiated from pANCAs occurring in non-UC patients by antigenic DNase sensitivity. In accordance with the presence invention, it has now been discovered that the pANCA of UC has immunoreactivity with

antigen located within the nuclear envelop of neutrophils. Accordingly, there is provided new methods of detecting pANCA associated with UC in a sample by detecting immunoreactivity with antigen located within the nuclear envelop of neutrophils.

Despite diligent efforts, attempts at isolating and identifying the structure of pANCA associated with UC have reportedly been unsuccessful. For the first time, pANCA associated with ulcerative colitis ("UCpANCA") has been recombinantly produced and characterized using the phage display technique, as described herein. Thus, in accordance with the present invention there is provided isolated, substantially purified and/or recombinantly produced UCpANCA and UCpANCA material and methods of recombinantly producing UCpANCA and UCpANCA material. In presently preferred embodiment, UCpANCA and UCpANCA material of the present invention is characterized as having immunoreactivity with antigen localized within the nuclear envelop of neutrophil, a perinuclear staining pattern by alcohol-fixed neutrophil indirect immunofluorescence assay, and immunoreactivity that is disrupted by pre-treatment of neutrophil with DNase.

The UCpANCA and UCpANCA material of the present invention is also characterized by the polynucleic acid sequences and amino acid sequences which may encode them. Exemplary sequence information is provided herein. Also provided are UCpANCA V_L and V_H polypeptides, UCpANCA polypeptide V_L segments and V_H segments, and polynucleic acids encoding these polypeptides. Exemplary complimentarity determining regions of these polypeptides are mapped in the sequence information provided herein.

This invention further provides methods for producing a libraries of phagemid expression vectors encoding heterodimeric antibody material of an immunoglobulin gene repertoire of pANCA seropositive ulcerative colitis, as well

as the libraries themselves. The present invention also provides methods for enriching such libraries to produce libraries of phagemid expression vectors encoding heterodimeric antibody material having immunoreactivity with UCpANCA antigen.

The phagemid expression vectors of the present invention can be encapsulated by phage particles or cells. Methods for expressing the encoded library or individual members of the encoded library as soluble or phage-anchored antibody material are also provided.

This invention also provides methods for detecting UCpANCA in a sample using UCpANCA and UCpANCA material of the present invention in immunoassays. Methods are also provided for using UCpANCA and UCpANCA material to isolate, characterize and clone UCpANCA antigen. Kits containing UCpANCA material are thus also provided.

For the first time, pANCA associated with ulcerative colitis ("UCpANCA") has been recombinantly produced and characterized using the phage display technique. Thus, in accordance with the present invention there is provided isolated, substantially purified and/or recombinantly produced UCpANCA and UCpANCA material. Also provided are polypeptides encoding UCpANCA V_L and V_H polypeptides, as well as UCpANCA V_L segments and V_H segments. Also provided are polynucleotides encoding these polypeptides.

This invention further provides methods for producing a libraries of phagemid expression vectors encoding heterodimeric antibody material of an immunoglobulin gene repertoire of pANCA seropositive ulcerative colitis, as well as the libraries themselves. The present invention also provides methods for enriching such libraries to produce libraries of phagemid expression vectors encoding heterodimeric antibody material having immunoreactivity with UCpANCA antigen.

The phagemid expression vectors of the present invention can be encapsulated by phage particles or cells. Methods for expressing the encoded library or individual members of the encoded library as soluble or phage-anchored antibody material are also provided.

This invention also provides methods for detecting UCpANCA in a sample using UCpANCA and UCpANCA material of the present invention in immunoassays and by localization of immunoreactivity of the sample within the nucleus of neutrophil. Methods are also provided for using UCpANCA and UCpANCA material to isolate, characterize and clone UCpANCA antigen. Kits containing UCpANCA material are thus also provided.

IV. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a reproduction of a photograph of cut-away confocal images depicting the location of immunoreactivity of UC$^+$ sera and EL-pANCA sera relative to the nucleus of neutrophil cells. The boundary of the nucleus and nuclear material is marked by reaction with propidium iodide (red) while the antibody-antigen reaction is marked in green. The left-hand view of the cut-away images (Fig. 1B and 1D) show both the antiserum (green) and propidium iodide (red) signals together, the middle view shows only the signal given by the antiserum reaction, and the right-hand view shows only the propidium iodide signal. The pattern generated using UC$^+$ serum (Fig. 1A and B) is clearly *within the nuclear border* and co-localizes with the outer edge of the propidium iodide-stained DNA. The antigenic target of EL-pANCA is clearly perinuclear since the signal is on the *outside of the nuclear border* and does not co-localize with propidium iodide-stained DNA.

Figure 2 is a reproduction of a photograph depicting the immunoreactivity of UC+ sera with nuclear antigen of neutrophil. UC+ sera was reacted with freeze dried and paraformaldehyde fixed neutrophil cells and the reaction examined by electron microscopy. Immunogold labeling was observed over heterochromatin DNA located on the inside periphery of the nucleus of neutrophils treated with UC+ sera (Figure 2A). As a comparison, the staining pattern of anti-histone (Figure 2C) and normal human serum (Figure 2B) on neutrophils prepared in the same manner is also depicted.

Figure 3 presents the amino acid sequences of the variable heavy chain domain of UCpANCA Fab clone 5-3 and 5-4 aligned with their human germ-line counterpart DP49. "-" indicates identity of amino acid residues.

V. DETAILED DESCRIPTION OF THE INVENTION

A. Definitions

As used herein the terms "isolated," "substantially pure," or "recombinant" in their various grammatical forms as a modifier of proteins including antibodies and antibody materials, polypeptides, amino acid sequences, polynucleotides, and nucleic acid sequences or molecules means that the proteins, polypeptides, amino acid sequences, polynucleotides, and nucleic acid sequences or molecules so designated have been produced in such form by the hand of man, and thus are separated from their native *in vivo* cellular environment. As a result of this human intervention, the isolated, pure and/or recombinant, proteins, polypeptides, amino acid sequences, polynucleotides, and nucleic acid sequences or molecules of the invention can be produced in large quantities and are useful in ways that the proteins, polypeptides, amino acid sequences, polynucleotides, and nucleic acid sequences or molecules as they naturally occur are not.

The terms "antibody" and "antibody molecule" in their various grammatical forms are used herein as collective nouns to refer to a population of immunoglobulin molecules which may be polyclonal or, more preferably, monoclonal in origin and which may be of any isotype, preferably of the gamma and kappa isotypes.

The phrases "monoclonal antibody" or "monoclonal antibody material" in its various grammatical forms refers to a population of antibody molecules or antibody material that contain only one species of idiotope capable of immunoreacting with a particular epitope on an antigen. A monoclonal antibody typically displays a single binding affinity for an epitope with which it immunoreacts; however, a monoclonal antibody may be a molecule having a plurality of idiotopes, each immunospecific for a different epitope, e.g., a bispecific monoclonal antibody.

The term "antibody material" in its various grammatical forms is used herein as a collective noun that refers to a population of immunologically active fragments of immunoglobulin molecules, i.e., molecules that contain an antibody combining site. Exemplary antibody materials of the present invention include those portions of immunoglobulin molecules known in the art as Fab, Fab', and $F(ab')_2$. An antibody combining site is that structural portion of the antibody molecule comprised of a heavy and light chain variable and hypervariable regions that specifically binds (immunoreacts with) an antigen.

The term "immunoreact" in its various grammatical forms means specific binding between an antigenic determinant-containing molecule, such as an antigen, and a molecule containing an antibody combining site such as an antibody molecule or antibody material.

The term "ANCA" refers to anti-neutrophil cytoplasmic antibody.

The term "pANCA" refers to ANCA having a cytoplasmic staining pattern with perinuclear highlighting, also referred to as a perinuclear staining pattern.

The term "UCpANCA" refers to an antibody associated with ulcerative colitis, that immunoreacts with nuclear antigen expressed by neutrophil, and produces a pANCA staining pattern in an alcohol-fixed neutrophil IIF assay.

The term "UCpANCA material" refers to isolated, substantially pure or recombinantly produced antibody material associated with ulcerative colitis, that immunoreacts with nuclear antigen expressed by neutrophil, and produces a pANCA staining pattern in an alcohol-fixed neutrophil IIF assay.

The term "UCpANCA polypeptides" refers to an isolated, substantially pure or recombinantly produced polypeptide contained as part of UCpANCA or UCpANCA material.

The phrase "pANCA seropositive ulcerative colitis" and the designation "UC+" are used as modifiers to indicate that the item modified tests positive in neutrophil ELISA or more preferably displays a pANCA staining pattern by alcohol-fixed neutrophil IIF assay.

The symbol "V_H" refers to immunoglobulin heavy chain variable domain which includes the variable segment ("V_H segment"), the diversity segment ("D") and the joining segment ("J_H segment").

The symbol "V_L" refers to immunoglobulin light chain variable domain which includes the variable segment ("V_L segment")and the joining segment ("J_H segment").

A "dimer" is polymer formed from two monomer molecules. When the dimer consists of two identical monomer molecules it will be referred to herein as "homodimeric." When a dimer consists of two distinct monomer molecules it will be referred to herein as "heterodimeric."

As employed herein, the term "substantially the same amino acid sequence" refers to amino acid sequences having at least about 80% identity with respect to the reference amino acid sequence, and preferably retaining comparable functional and biological properties characteristic of the polypeptide defined by the reference amino acid sequence. Preferably, polypeptides having "substantially the same amino acid sequence" will have at least about 90%, more preferably 95% amino acid identity with respect to the reference amino acid sequence; with greater than about 97% amino acid sequence identity being especially preferred.

B. Identifying a Relevant Population of Lymphocytes for the Production of a UC Immunoglobulin Gene Repertoire

An immunoglobulin gene repertoire is a collection of different gene segments from the immunoglobulin gene complex, and may be isolated from natural sources or can be generated artificially. Natural sources of immunoglobulin gene repertoires are typically a heterogenous population of antibody producing cells, i.e., B lymphocytes (B cells). However, in order to bias an immunoglobulin gene repertoire so that it represent the immunoglobulin gene segments associated with a particular disease or a particular disease subset, a disease associated population of rearranged B cells must first be generated or isolated. If, for example, the disease is associated with one or more antigens, a population of B cells enriched for genetic material producing antibodies having affinity for the antigen(s) can be generated by repeated immunization of a healthy animal with the antigen(s) before collecting the rearranged B cells. If, for example, antigen(s) associated with the disease are not known or have not been isolated, a population of rearranged B cells from a diseased individual can be collected from the blood.

Prior to the present invention, significant obstacles existed to the production of a useful immunoglobulin gene repertoire representative of the immunoglobulin gene segments associated with pANCA seropositive ulcerative colitis (referred to herein as an "immunoglobulin gene repertoire of UC+") using either of these methods. First, according to the published literature, there had been no antigens identified or isolated that could be used to generate an enriched population of B cells by immunization. In addition, as demonstrated by the failure in the prior art to isolated or produce UCpANCA from blood lymphocytes, there had been no known population of B cells having represented therein a sufficient quantity of B cells producing UCpANCA to be useful for generating an immunoglobulin gene repertoire of UC+. The present invention has overcome this significant obstacle by identifying, for the first time, a population of B cells producing UCpANCA.

The B cell origin of UCpANCA could have been either B cells of the systemic immune system or B cells associated with specific tissue. Accordingly, peripheral blood lymphocytes (PBL), mesenteric lymph-node lymphocytes (MNL), and lamina propria lymphocytes (LPL) were isolated from pANCA seropositive UC patients to determine which lymphocytes, if any, produced UCpANCA.

1. Peripheral Blood Lymphocytes of UC+

Peripheral blood lymphocytes (PBL) were isolated directly by Ficoll-Hypaque fraction from 17 UC patients. All 17 of these UC patients were seropositive for pANCA by neutrophil ELISA, 16 of which demonstrated a pANCA staining pattern and the other displayed a cANCA staining pattern by the fixed neutrophil indirect immunofluorescence assay (IIF assay).

Immunoglobulin spontaneously produced by these PBL was generated by extensively washing isolated PBL and culturing them at 37°C in a humidified atmosphere of 5% CO_2:95% air for 12 days at a concentration of 2 x 10^6 cells/ml in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics as described by MacDermott, R.P., et al., Gastroenterology 81:844-852 (1981), incorporated herein by reference. Supernatants from these cultures were analyzed for IgG content by solid phase radioimmunoassay as described by MacDermott, R.P., et al., Gastroenterology 81:844-852 (1981).

The neutrophil ELISA was used to detect ANCA in 12 day PBL culture supernatant diluted 1:2 in blocking buffer and the IIF assay using undiluted supernatant was used to characterize the ANCA staining pattern as pANCA or cANCA. A comparison to the serum tests is provided in Table 1.

Table 1: pANCA in supernatants of peripheral blood lymphocytes from ELISA positive UC patients.

n	Serum		PBL Supernatant	
	# ELISA Positive	# ELISA Positive & Perinuclear	# ELISA Positive	# ELISA Positive & Perinuclear
17	17	16	2	1

Only two (2/17) PBL samples were found to spontaneously express ANCA but only one of these displayed a pANCA staining pattern. When seven of the PBL samples were cultured in the presence of IL-4 (5 ng/ml) and anti-CD40 antibody (1 μg/ml) to determine if UCpANCA production could be stimulated, none of the seven samples could be stimulated to produce a pANCA staining pattern. Significantly, then, B cells spontaneously secreting UCpANCA could not be found in the PBL fraction of pANCA seropositive UC patients, nor

could PBL cells be stimulated to produce UCpANCA using a combination of stimuli known to augment IgG_1 production.

2. Mesenteric Lymph-node Lymphocytes of UC+

Mesenteric lymph-node lymphocytes (MNL) were isolated from five of the same 17 UC patients from which PBL samples were obtained. All five of these UC patients were seropositive for pANCA by neutrophil ELISA, four of which displayed pANCA staining pattern and one of which displayed a cANCA staining pattern by IIF assay.

The neutrophil ELISA was used to detect ANCA in MNL culture supernatant diluted 1:2 in blocking buffer and, if positive for ANCA, the IIF assay using undiluted supernatant was used to characterize the ANCA staining pattern as pANCA or cANCA. Cultured MNL cells did not spontaneously produce ANCA. However, in a preliminary experiment, cultured MNL from two of these UC patients could be stimulated to produce ANCA having a pANCA staining pattern by incubation with IL-4 (5 ng/ml) and anti-CD40 antibody (1 μg/ml) suggesting the presence of primed but not previously activated autoimmune B cells in this compartment.

3. Lamina Propria Lymphocytes of UC+

Lamina propria lymphocytes (LPL) from human intestinal mucosa of colonic surgical specimens of disease-involved and uninvolved tissue from UC and CD patients as well as patients with diverticulitis and normal mucosa (the latter two groups were termed "non-IBD patients") were isolated as previously described by Bull, D.M. and Bookman, M.A., J. Clin. Invest. 59:966-974 (1977) as modified by MacDermott, R.P., et al., Gastroenterology 78:47-56 (1980) and MacDermott, R.P., et al., Gastroenterology, 81:844-52 (1981), all of which are incorporated herein by reference. In brief, mucosa was dissected free of muscularis and washed

in HEPES buffered calcium-magnesium free Hank's balanced salt solution containing 5% human serum and antibiotics (wash buffer). After weighing and mincing, the 2-5 mm mucosal pieces were stirred with 0.75mM EDTA containing wash buffer for 45 minutes at 37°C. This treatment was repeated until no more crypt cells were released in the wash solution. The mucosal pieces were digested by collagenase (16U/ml, Worthington Biochemical, Freehold, NJ) in wash buffer supplemented with 10% human serum, with constant stirring at 37°C in a humidified atmosphere of 5% CO_2:95% air. LPL were separated from the digestion supernatant by Ficoll-Hypaque gradient centrifugation as previously detailed by Saxon, A.F., et al., J.Allergy Clin. Immunol., 86:202-210 (1990), incorporated herein by reference.

Isolated LPL were washed extensively and cultured as described above for PBL to generate spontaneous production of immunoglobulin. Supernatants from these cultures were analyzed for IgG content by solid phase radioimmunoassay as described by MacDermott, R.P., et al., Gastroenterology 81:844-852 (1981). The neutrophil ELISA was used to detect ANCA in 12 day PBL culture supernatant diluted 1:2 in blocking buffer and the IIF assay using undiluted supernatant was used to characterize the ANCA staining pattern as pANCA or cANCA.

The results, as set forth in Table 2 below, demonstrate that 68% (15/22) of all supernatants derived from LPL of UC patients are ANCA positive with 71% (12/17) of involved and 60% (3/5) of uninvolved mucosa expressing ANCA.

Table 2. Summary of alcohol fixed neutrophil ELISA data and correlative IIF staining pattern of cultured LPL supernatants

Mucosal Type	n	ANCA Positive n(% of samples)	IIF Pattern n (% of pos. samples)		IgG Level (μg/ml)
			p-ANCA	c-ANCA	
UC	22†	15(68%)*	9(60%)	6 (40%)	25x/+ 28
-involved	17	12(71%)*	8(67%)	4 (33%)	31x/+ 30
-uninvolved	5	3(60%)**	1(33%)	2 (67%)	12x/+ 16
CD	8	1(13%)	0 (0%)	1(100%)	15x/+ 26
Non-IBD	15	2(13%)	0 (0%)	2(100%)	5x/+ 9
-diverticulitis	9	1(11%)	0 (0%)	1(100%)	3x/+ 6
-normal	6	1(16%)	0 (0%)	1(100%)	10x/+ 11

* $p<0.01$ vs. non-IBD and $p<0.02$ vs. CD

** $p<0.1$ vs. non-IBD: The % ANCA positively of uninvolved samples from UC patients is not significantly different from that of the non-IBD patients.

† The number (n) of samples assayed for the UC population is one greater than the actual number of patients (21) participating in the study since two samples (involved and uninvolved) were obtained from one of the patients.

The small sample size of uninvolved mucosa from UC patients precluded the statistical significance of that ANCA expression. In contrast, only 13% of culture supernatants derived from both CD (1/8) and non-IBD LPL (2/15) expressed ANCA ($P<0.02$ and $P<0.01$ respectively vs. UC). Sixty percent (9/15) of the ANCA positive LPL of UC patients displayed a pANCA staining pattern. All of the ANCA-expressing supernatants from non-UC LPL displayed a cANCA staining pattern.

Since an increase in the spontaneous production of total IgG by LPL from UC patients has been reported, it might be expected that the presence of ANCA in UC

supernatants is due to this increase in IgG. Therefore total IgG was measured in each LPL culture supernatant (UC, CD, and non-IBD patients) and plotted against the level of binding by neutrophil ELISA using linear regression analysis. No correlation was found between the level of total IgG in the LPL supernatants and the level of ANCA binding. The finding of ANCA positivity in LPL of UC patients is not simply due to enhanced levels of IgG in UC* LPL supernatants.

Finally, LPL were also isolated from the five UC patients that the PBL and MNL were isolated as described above, allowing direct comparison of ANCA expression and staining pattern between these three cell types from the same individuals. Again, four of these five patients (4/5) were seropositive for ANCA and all but one displayed a pANCA staining pattern while the other was cANCA. The LPL were cultured as described herein and the supernatants tested by neutrophil ELISA and IIF assay for ANCA and ANCA staining pattern. The results of those assays and the data from the same assays as performed on serum, PBL supernatant, and MNL supernatant is reported in Table 3.

Table 3: Correlation of pANCA in supernatants of lamina propria lymphocytes (LPL) peripheral blood lymphocytes (PBL) and mesenteric nodal lymphocytes (MNL) with serum p-ANCA values from ELISA positive UC patients

Patient #	Serum		LPL		PBL		MNL	
	% of Pos.	IF Pattern	% of Pos.	IF Pattern	% of Pos.	IF Pattern	% of Pos.	IF Pattern
1	91(+)	P	18(+)	P	4(-)	n/a	2(-)	n/a
2	61(+)	P	13(+)	P	8(-)	n/a	4(-)	n/a
3	24(+)	P	21(+)	P	2(-)	n/a	1(-)	n/a
4	81(+)	P	5(-)	n/a	7(-)	n/a	2(-)	n/a
5	17(+)	C	3(-)	n/a	17(+)	C	5(-)	n/a

(+) = ELISA positive sample (greater than the mean plus two standard deviations of ELISA values for supernatants of normal LPL)
(-) = ELISA negative sample (less than the mean plus two standard deviations of ELISA values for supernatants of normal LPL)
P = Perinuclear immunofluorescence pattern
C = Cytoplasmic immunofluorescence pattern
n/a = not applicable in ELISA negative samples

Three of the 4 (75%) LPL supernatants from pANCA seropositive UC patients expressed ANCA and displayed a pANCA staining pattern identical to that of its matched serum ANCA and only one (1/4) of the LPL supernatants was negative for ANCA while the corresponding serum showed a positive pANCA reaction.

Accordingly, it has been discovered that a population of B cells secreting UCpANCA are present and can be isolated from the mucosal LPL fraction of patients diagnosed with pANCA seropositive ulcerative colitis for use in generating libraries of the immunoglobulin gene repertoire of UC. These ANCA secreting B cells are present in both disease-involved (71%) and uninvolved (60%) mucosa. IIF analysis showed that the majority of these ANCA (60%) are pANCA (Table 2). In contrast only 13% of CD and non-IBD inflammatory/non-inflammatory LPL produced ANCA after 12

days of culture. None of these displayed pANCA staining pattern (Table 2).

In accordance with the present invention, lymphocytes from the lamina propria of a human patient diagnosed with UC and seropositive for pANCA, preferably lymphocytes form an inflamed region of the lamina propria, are used to generate a library of the human immunoglobulin gene repertoire of UC^+. It should be noted that the greater the genetic heterogeneity of the population of B cells form which the gene segments of the immunoglobulin gene repertoire are obtained, the greater the diversity of the immunological repertoire that will be made available for screening according to the methods of the present invention. Thus, B cell from the lamina propria of different individuals, particularly those having an immunologically significant age difference, and cells from individuals from different families and different races can be combined to increase the heterogeneity of a repertoire. Immunoglobulin gene repertoires of UC^+ can be derived from LPL producing immunoglobulin having heavy chains of the IgA, IgD, IgE, IgG, or IgM isotypes, most preferably from LPL producing immunoglobulin having heavy chains of the IgG or IgM isotype, and even more preferably from LPL producing immunoglobulin having heavy chains of the IgG_1 isotype. Immunoglobulin gene repertoires of UC^+ can be derived from LPL producing immunoglobulin having light chains of the kappa or lambda isotype, preferably from LPL producing immunoglobulin having light chains of the kappa isotype.

C. Localization of UCpANCA Antigen

The second obstacle which had to be overcome to isolate UCpANCA was to determine how it could be segregated from antibody material generated from the immunoglobulin gene repertoire of UC^-. The isolation and identification of the

antigen(s) specifically recognized by UCpANCA has not yet been reported in the literature.

The conventional assay for detecting serum UCpANCA is by the IIF assay using cytocentrifuged, alcohol-fixed neutrophils. As discussed above, the typical patterns produced by ANCAs using alcohol-fixed neutrophils are cANCA and pANCA. Unfortunately, these staining patterns can be generated by more than one species of ANCA such as, for example, ANCA specific for elastase (EL-pANCA) and ANCA specific for myeloperoxidase (MPO-pANCA). Although UCpANCA could be distinguished from these other pANCAs by its failure to immunoreact with elastase or myeloperoxidase, additional assays that would allow discrimination between such non-UCpANCA material and UCpANCA material was desired.

One such assay employed herein is referred to as the "DNase-sensitivity assay." It has been shown that the pANCA staining pattern of UCpANCA but not the pANCA staining pattern of non-UCpANCA is abolished or becomes cANCA when the neutrophil are pre-treated with DNase. Accordingly, in addition to the neutrophil ELISA and the conventional IIF assay, the DNase-sensitivity assay was also used to identify and isolate UCpANCA.

Another means for overcoming the shortcoming of the conventional IIF assay as a means for detecting UCpANCA is the localization of the UCpANCA antigen as described for the first time herein. The staining patterns generated by the IIF assay do not always accurately reflect the cellular localization of the reactive antigens. For example, it is known that some cytoplasmic antigens artifactually associate with the neutrophil nucleus after alcohol fixation to yield a "perinuclear" staining pattern. In order to determine whether the UC specific pANCA reactive antigen might, in fact, reside in some aspect of the nuclear domain or whether the apparent perinuclear localization was an artifact of the

alcohol fixation of neutrophils, the location of binding within the neutrophil by IgG from sera of pANCA seropositive UC patients was examined by both confocal laser microscopy and immune electron microscopy using two methods of non-alcohol cell fixation. By confocal microscopy the majority of UC+ sera examined displayed a nuclear reaction which localized to the inner side of the nuclear (membrane) periphery. Immune electron microscopy revealed that binding was localized predominantly over heterochromatin concentrated toward the periphery of the nucleus. This reaction, however, was not due to antibody recognition of DNA since these sera did not react in a (double stranded) DNA ELISA.

1. Alcohol-Fixed Neutrophil IIF Assay

A panel of sera from twenty-five patients diagnosed with UC and previously determined to express moderate to high levels of ANCA (range of neutrophil binding levels 37%-153%) in the neutrophil ELISA was further examined by the IIF assay to determine the type of staining pattern each displayed. All (100%) of the ANCA containing sera displayed a pANCA staining pattern. This sera was also confirmed to be negative for antibodies that recognized double stranded (ds) DNA using the anti-dsDNA assay kit from HELIX Diagnostics (West Sacramento, CA) in accordance with the manufacturer's directions.

Similarly, sera previously characterized as containing antibodies against myeloperoxidase and elastase (kindly provided by Dr. J. Charles Jennette of the University of North Carolina located in Chapel Hill, NC were subjected to the same IIF assay and yielded a typical pANCA staining pattern although the antigens recognized by these latter antibodies are known to be constituents of cytoplasmic granules.

2. Paraformaldehyde-fixed Neutrophil IIF Assay

It has been reported that when neutrophils are fixed by non-alcohol based reagents (e.g., paraformaldehyde, formalin, etc.), the perinuclear staining pattern obtained with either MPO-pANCA sera or EL-pANCA sera is abolished and converted to a more cytoplasmic staining pattern. The reaction of UC* sera as well as MPO- and EL-pANCA sera were examined using paraformaldehyde/acetone fixed neutrophils previously allowed to settle onto the slide. This method of slide preparation appeared to eliminate the redistribution of nuclear material that occurs due to cytocentrifugation and maintained the three dimensional morphology of the cells.

The reactions were visualized by confocal IIF microscopy. The DNA specific fluorescent dye, propidium iodide, was used to delineate the boundary of the nuclear material for reference. The staining pattern observed in paraformaldehyde fixed neutrophils treated with MPO-pANCA sera was a combination of granular cytoplasmic with perinuclear highlighting staining while the EL-pANCA sera retained a thin perinuclear staining pattern. A relatively broad band of staining around the periphery of the nucleus was observed in paraformaldehyde fixed neutrophils treated with UC* serum while serum from a normal donor was negative.

3. Comparison of UC* and EL-pANCA Staining Patterns by Confocal Microscopy

Since the "perinuclear" staining reaction of both UC* sera and EL-pANCA sera was maintained in the paraformaldehyde fixed neutrophils, cut-away confocal images were examined and compared to determine whether the nuclear location of the antigenic sites recognized by these two antisera were similar or different. As depicted in Figure 1, the boundary of the nucleus and nuclear material was

marked by reaction with propidium iodide (red) while the antibody-antigen reaction was marked in green. The left-hand view of the cut-away images (Figure 1B and 1D) show both the antiserum (green) and propidium iodide (red) signals together, the middle view shows only the signal given by the antiserum reaction, and the right-hand view shows only the propidium iodide signal.

The pattern generated using UC* serum (Fig. 1A and B) is clearly *within the nuclear border* and co-localizes with the outer edge of the propidium iodide-stained DNA. The antigenic target of EL-pANCA is clearly perinuclear since the signal is on the *outside of the nuclear border* and does not co-localize with propidium iodide-stained DNA.

4. Electron Microscopic Localization of UCpANCA Antigen

To verify that the antigen(s) recognized by the UC* sera was localized in the nucleus and that this localization was different from that of the EL-pANCA specific antigen, freeze dried and paraformaldehyde fixed cells were reacted with UC* sera and the reaction examined by electron microscopy. Immunogold labeling was observed over heterochromatin DNA located on the inside periphery of the nucleus of neutrophils treated with UC* sera (Figure 2A). To assure that the nuclear localization of the UCpANCA antigen was not an artifact of the cell preparation procedure, the staining pattern of anti-histone and normal human serum was also examined. As expected, no significant reaction was observed in neutrophils treated with normal human serum (Figure 2B) while immunogold gold labeling was observed over the entire nucleus in neutrophils treated with the anti-histone serum (Figure 2C). These findings confirm the nuclear localization of UCpANCA antigen as demonstrated using confocal microscopy.

5. Confocal Microscopic Analysis of a Panel of UCpANCA Positive Sera

To determine whether all or only some UC+ patient sera immunoreact with nuclear antigen(s), a panel of sera from the twenty five pANCA seropositive UC patients was further analyzed by confocal microscopy using paraformaldehyde-acetone fixed neutrophils. As seen in Table 4, 88% (22/25) of the UC+ sera tested yielded a nuclear reaction.

Table 4. Confocal Microscopic Analysis of Staining Patterns of pANCA expressing UC Sera.

Nuclear		Cytoplasmic
22/25 (88%)		3/25 (12%)
Nuclear Central	**Nuclear Periphery***	
4/25 (16%)	18/25 (72%)	

* staining localized to the nuclear periphery but inside the boundary of the nuclear membrane

Of these, 18/25 (72%) were found to localize to the inner side of the nuclear periphery and 4/25 (16%) were found in a more central nuclear location. Only 12% (3/25) of the sera yielded a cytoplasmic reaction. These results indicate that the majority (88%) of UCpANCA recognize an antigenic specie(s) located in the neutrophil nucleus possibly in association with DNA. This finding renders the UCpANCA specific antigen unique among the thus far described ANCA antigens and provided a unique and reliable basis for discriminating UCpANCA material from non-UCpANCA material.

Thus, in accordance with the present invention, there is provided a new and useful method of detecting UCpANCA in a sample, comprising (a) contacting the sample and a detectable secondary reagent with fixed neutrophil under

conditions suitable to form an immune complex of neutrophil, UCpANCA and detectable secondary reagent, wherein said secondary reagent has binding specificity for UCpANCA or the class determining portion of UCpANCA; (b) separating unbound secondary reagent from immune complex; and (c) assaying for the presence or absence of UCpANCA containing immune complex within the nucleus of the neutrophil by detecting the presence or absence of bound secondary reagent. UCpANCA is considered present in the test sample if UCpANCA-containing immune complex is detected within the neutrophil nucleus, or more preferably associated with heterochromatin DNA located on the inside periphery of the nucleus of neutrophils.

The assays of the present invention may be forward, reverse or simultaneous as described in U. S. Patent No. 4,376,110, issued March 8, 1983 to David et al., incorporated herein by reference in its entirety. In the forward assay, each reagent is sequentially contacted with fixed neutrophil. If desired, separation of bound from unbound reagent can be accomplished before the addition of the next reagent. In a reverse assay, all reagents are pre-mixed prior to contacting fixed neutrophil. A modified method of a reverse assay is described in U.S. Patent No. 4,778,751 issued October 18, 1988 to El Shami et al., incorporated herein by reference in its entirety. In a simultaneous assay, all reagents are separately but contemporaneously contacted with the fixed neutrophil.

A sample can be obtained from any biological fluid, for example, whole blood, plasma, or other bodily fluids or tissues having UCpANCA, preferably serum or supernatant of LPL.

The separation steps for the various assay formats described herein, including removing unbound secondary reagent from the immune complex, can be performed by methods known in the art. When appropriate, a simple washing with a

suitable buffer followed by filtration or aspiration is sufficient. If the neutrophil(s) is immobilized on a particulate support it may be desirable to centrifuge the particulate material, followed by removal of wash liquid. If the neutrophil(s) is immobilized on membranes or filters, applying a vacuum or liquid absorbing member to the opposite side of the membrane or filter allows one to draw the wash liquid through the membrane or filter.

The methods of the present invention are normally carried out at room temperature and 37°C. Accordingly, temperatures suitable for performing the methods of the present invention generally range from about 22°C to about 38°C.

In accordance with the methods of the present invention, neutrophil(s) can be fixed by methods well known in the art which render the neutrophils permeable to the reagents used in the methods of the present invention. Suitable fixatives include, for example, methanol, ethanol, formalin, or the like and preferably include non-alcohol fixatives such as, for example paraformaldehyde and acetone. Of course, one of skill in the art will appreciate that such fixatives should not substantially alter nuclear or cellular morphology of the neutrophil(s).

Neutrophil(s) and secondary reagents appropriate for use in the practice of the present invention will depend upon the origin of the sample assayed. As used herein, the terms "patient," "subject," or "individual" when referring to the origin of the sample to be assayed, means any animal capable of producing UCpANCA, including for example, humans, non-human primates, rabbits, rats, mice, and the like. Preferably, neutrophils and secondary reagents employed will have specific reactivity for the species from which the sample to be tested is obtained. For example, to assay for UCpANCA in a sample obtained from a human subject, the

neutrophils and the secondary reagent are preferably specific for humans. If multiple secondary reagents are used, for example secondary antibodies, each antibody is preferably species-specific for its antigen.

Neutrophils useful in the present invention can be obtained from a variety of sources, e.g., the blood of a human, non-human primates, rabbits, rats, mice, and the like, by methods known to those of skill in the art.

The term "secondary reagent" as used herein, refers to any reagent or combination of reagents that can bind UCpANCA. For example, a secondary reagent can be an anti-UCpANCA antibody or fragments thereof specific for any idiotope of UCpANCA, but preferably not one that would be competitive with neutrophil binding or cause stearic hindrance of neutrophil/UCpANCA binding. Alternatively, a secondary reagent can be an anti-isotype antibody having specificity for a class determining portion of UCpANCA, or can be protein A or protein G.

Secondary reagents useful in the practice of the present invention can be obtained by techniques well known in the art or from any one of several commercial sources. If antibodies are used, they are preferably monoclonal or pooled monoclonal.

Another alternative for increasing the sensitivity of the assay of the present invention is to use a multiple antibody system for the secondary reagent. Thus, the methods of the present invention may be performed using a combination of antibodies as the secondary reagent, wherein at least one secondary antibody reagents of the combination has specificity for UCpANCA or the class determining portion of UCpANCA and at least one secondary antibody of the combination is detectable.

The term "detectible secondary reagent" refers to secondary reagent, as defined above, that can be detected or

measured by a variety of analytical methods. This term includes reagents that are directly detectible without attachment of signal generating labels, or those that can be labeled with a signal generating system to permit detection or measurement, such as, for example, a radioisotope, chromogenic or fluorogenic substance, a chemiluminescent marker, gold, or the like. In any of the above methods, the reactivity of the secondary reagent with the UCpANCA should not be significantly altered by the presence of the label.

In a presently preferred embodiment the secondary reagent is anti-IgG antibody material rendered detectable by chemically linking it to a fluorogenic compound. Suitable fluorogenic compounds are those that emit light in ultraviolet or visible wavelength subsequent to excitation by light or other energy source. The fluorogens can be employed alone or with a suitable quencher molecule. The methods of conjugating suitable fluorogens have been reported and are described, for example, in Methods in Enzymology, Volume 74, Part C, 32105 (Van Vunakis and Langone, Editors 1991). Alternatively, secondary antibody linked to fluorogen useful for the practice of the present invention may be obtained from one of any number of commercially available sources. In yet another preferred embodiment the secondary reagent is gold labeled protein A or protein G.

Depending on the nature of the label used, a signal can be detected, for example, by irradiating the complexed test sample with light and observing the pattern of fluorescence; by electron microscopy; or, in the case of chemiluminescence or a radioactive label, by employing a radiation counter such as a gamma counter or gamma emitting markers such as iodine-125.

D. Production of Immunoglobulin Gene Repertoire of UC*

Methods for preparing fragments of genomic DNA from which immunoglobulin variable region genes can be cloned as a diverse population are well known in the art. See, for example, Herrmann et al., Methods in Enzymology, 152: 180-183 (1987); Frischauf, Methods in Enzymology, 152: 183-190 (1987); Frischauf, Methods in Enzymology, 152: 190-199 (1987); and DiLella et al., Methods in Enzymology, 152: 199-212 (1987). (The teachings of the references cited herein are hereby incorporated by reference.)

An immunoglobulin gene repertoire can be isolated either from genomic material containing the genes expressing the V, D and J segments of immunoglobulin variable domains or from the messenger RNA (mRNA) which represents the transcript of the variable domain. The difficulty in using the genomic DNA from other than non-rearranged B lymphocytes is in juxtapositioning the sequences coding for the V_H, D, and J_H segments of the heavy chain variable domain with one another and in juxtapositioning the sequences coding for the $V_{K/\lambda}$ and $J_{K/\lambda}$ segments of the light chain variable domain with one another where the sequences are separated by introns. The sequences containing the proper exons must be isolated, the introns excised, and the exons spliced in proper order and orientation. For the most part, this will be difficult, so the alternative technique employing rearranged B cells will be the method of choice because the V_H, D, and J_H segments or $V_{K/\lambda}$ and $J_{K/\lambda}$ segments have translocated to become adjacent, so that the sequence is continuous (free of introns) for the entire variable regions.

When mRNA is utilized the B cells will be lysed under RNase inhibiting conditions. In one embodiment, the first step is to isolate total cellular RNA. Poly A+ mRNA can

then be selected by hybridization to oligo -dT cellulose column. The presence of mRNA coding for the heavy and/or light chain polypeptides can then be assayed by hybridization with single stranded DNA of the appropriate genes. Conveniently, the sequences coding for the constant portion of immunoglobulin heavy and light chains can be used as polynucleotide probes, which sequences can be obtained from available sources. See for example, Early and Hood, Genetic Engineering, Setlo and Hollaender, eds., Vol. 3, Plenum Publishing Corporation, New York (1981), pages 157-188; and Kabat et al., Sequences of Immunological Interest, National Institutes of Health, Bethesda, Maryland (1987).

In a presently preferred embodiment, total RNA is extracted from LPL cells of UC* patients and the RNA preparation enriched for immunoglobulin heavy and light chain coding mRNA. Enrichment is typically accomplished by subjecting the total RNA preparation, or a partially purified mRNA product thereof, to a primer extension reaction employing a polynucleotide synthesis primer as described herein. Exemplary methods for producing V_H and V_L gene repertoires using polynucleotide synthesis primers are described in PCT Application No. PCT/US90/02836 (International Publication No. WO 90/14430). Particularly preferred methods for producing a gene repertoire rely on the use of preselected oligonucleotides as primers in a polymerase chain reaction (PCR) to perform PCR reaction products as described herein.

1. Preparation of Primers for Producing Immunoglobulin Gene Repertoires of UC*

It is preferred that V_H and V_L immunoglobulin gene repertoires of UC* are prepared separately prior to their utilization in the present invention. Repertoire preparation is typically accomplished by primer extension,

preferably by primer extension in a polymerase chain reaction (PCR) format.

To produce a repertoire of V_H-coding DNA homologs by primer extension, the nucleotide sequence of a primer is selected to hybridize with a plurality of immunoglobulin heavy chain genes at a site substantially adjacent to the V_H-coding region so that a nucleotide sequence coding for a functional (capable of binding) polypeptide is obtained. To hybridize to a plurality of different V_H-coding nucleic acid strands, the primer must be a substantial compliment of a nucleotide sequence conserved among the different strands. Preferred sites include nucleotide sequences in the constant region, the leader region and the promoter region, although fragments of the V_H domain can be obtained by using sites in the variable domain framework regions, J region and the like.

If the repertoires of V_H-coding and V_L-coding DNA homologs are to be produced by (PCR) amplification, two primers, i.e., a PCR primer pair, must be used for each coding strand of nucleic acid to be amplified. In PCR, each primer works in combination with a second primer to amplify a target nucleic acid sequence. The choice of PCR primer pairs for use in PCR is governed by considerations as discussed herein for producing immunoglobulin gene repertoires. That is, the primers have a nucleotide sequence that is complimentary to a sequence conserved in the repertoire. Primer sequences useful for amplification of the V_H-coding and V_L-coding DNA homologs are shown in SEQ ID NOs: 5 through 16.

2. Polymerase Chain Reaction to Produce V_H and V_L Gene Repertoires of UC

The strategy used for cloning the V_H and V_L genes contained within a repertoire will depend, as is well known

in the art, on the type, complexity, and purity of the nucleic acids making up the repertoire. Other factors include whether or not the genes are contained in one or a plurality of repertoires and whether or not they are to be amplified and/or mutagenized.

V_H and V_L gene repertoires are comprised of polynucleotide coding strands, such as mRNA and/or the sense strand of genomic DNA. If the repertoire is in the form of double-stranded genomic DNA, it is usually first denatured, typically by melting, into single strands. A repertoire is subjected to a PCR reaction by treating (contacting) the repertoire with a PCR primer pair, each member of the pair having a preselected nucleotide sequence. The PCR primer pair is capable of initiating primer extension reactions by hybridizing to nucleotide sequences, preferably at least about ten nucleotides in length and more preferably at least about twenty nucleotides in length, conserved within the repertoire. The first primer of a PCR primer pair is sometimes referred to herein as the "sense" primer because it hybridizes to the coding or sense strand of a nucleic acid. In addition, the second pair of the PCR primer pair is sometimes referred to herein as the "anti-sense primer" because it hybridizes to a non-coding or anti-sense strand of a nucleic acid, i.e., a strand complimentary to a coding strand.

In a presently preferred embodiment, total RNA from LPL of UC+ human patients is utilized to generate a plurality of V_H- and V_L-encoding DNA homologs. Since serum UCpANCA is typically of the IgG_1 and kappa isotypes, variable heavy chain and kappa chain family-specific PCR primers are preferably paired with PCR primers that hybridize to $\gamma 1$ and κ constant regions, respectively. Preferred PCR primers for generating V_H- and V_L-encoding DNA homologs of the

immunoglobulin gene repertoire of UC* are provided in SEQ ID NOs. 5 through 16.

The PCR reaction is performed by mixing the PCR primer pair, preferably a predetermined amount thereof, with the nucleic acid of the repertoire, preferably a predetermined amount thereof, in a PCR buffer to form a PCR reaction admixture. The admixture is maintained under polynucleotide synthesizing conditions for a time period, which is typically predetermined, sufficient for the formation of a PCR reaction product, thereby producing a plurality of different V_H-coding and/or V_L-coding DNA homologs.

A plurality of first primer and/or a plurality of second primers can be used in each amplification, e.g., one species of first primer can be paired with a number of different second primers to form several different primer pairs. Alternatively, an individual pair of first and second primers can be used. In any case, the amplification products of amplifications using the same or different combinations of first and second primers can be combined to increase the diversity of the gene library.

In a presently preferred embodiment, V_H-encoding DNA homologs are created in seven separate reactions pairing one of the heavy chain variable segment family specific primers (SEQ ID NOs: 6 through 12) with the γ1 constant region specific primer. Equal quantities of each of the homologs produced from each of the seven reactions are then combined to create a V_H-encoding DNA homolog library of the immunoglobulin gene repertoire of UC* ("V_H library of UC*"). Likewise, it is presently preferred that V_L-encoding DNA homologs are created in three separate reactions pairing one of the kappa light chain variable segment family specific primers (SEQ ID NOs: 14 through 16) with the κ constant region specific primer. Equal quantities of the homologs produced from each of the three reactions are then combined

to create a V_L-encoding DNA homolog library of the immunoglobulin gene repertoire of UC$^+$ ("V_L library of UC$^+$").

PCR amplification methods are described in detail in U.S. Patent NOs. 4,683,195, 4,683,202, 4,800,159, and 4,965,188, and at least in several texts, including "PCR Technology: Principles and Applications for DNA Amplification," H. Erlich, ed., Stockton Press, New York (1989); and "PCR Protocols: A Guide to Methods and Applications," Innis et al., eds., Academic Press, San Diego, California (1990).

E. Production of a Library Encoding Heterodimeric Antibody Material from V_H- and V_L-coding DNA homologs of the Immunoglobulin Gene Repertoire of UC$^+$

1. General Rationale

Methods of generating UCpANCA material of the present invention using the phage display technique preferably first include combining the V_H and V_L libraries of UC$^+$ to form a "heterodimeric library of UC$^+$" that encodes heterodimeric antibody material from the immunoglobulin gene repertoire of UC$^+$. Members of the heterodimeric library of UC$^+$ can then be expressed in an *in vitro* expression host such as, for example, *E. coli*, so that co-expressed V_H and V_L polypeptides may be assembled into functional heterodimeric antibody material. Next, members of the heterodimeric library of UC$^+$ are screened for the ability of their expressed heterodimeric antibody material to bind UCpANCA antigen. This screening process requires a means of linking expression product (i.e., heterodimeric antibody material) with the V_H and V_L DNA homologs that encode it. This is accomplished by anchoring the heterodimeric antibody material to a phage coat which in turn encapsulates the V_H and V_L DNA homologs encoding the heterodimeric antibody material. Finally, heterodimeric library members of UC$^+$

that encode heterodimeric antibody material having antigen binding capacity are segregated from the remainder of the library for additional characterization and/or use.

2. Vectors for Expression of Heterodimeric Antibody Material on the Surface of Phage

Since the objective is to achieve expression of the combined V_H and V_L libraries of UC* in a manner which links expression product with the V_H and V_L DNA homologs which encode it, it is expeditious to create and store the heterodimeric libraries of the present invention in a suitable phagemid expression vector. Phagemid expression vectors useful in the practice of the present invention include monocistronic vectors and, more preferably, dicistronic vectors.

Phagemid vectors for expression of a heterodimeric antibody material on the surface of a filamentous phage particle are recombinant DNA molecules adapted for receiving V_H- and V_L-encoding DNA homologs and expressing these homologs as fusion proteins wherein one of these homologs is fused to a filamentous phage coat protein membrane anchor domain and to a prokaryotic secretion signal domain, and the other of these homologs is fused to a prokaryotic secretion signal domain. That is, one of either the V_H and V_L polypeptides is expressed as a fusion protein including a filamentous phage coat membrane anchor and a prokaryotic secretion signal, and the other polypeptide is expressed as a fusion protein including a prokaryotic secretion signal. A prokaryotic secretion signal is relatively short amino acid sequence at the amino end of a polypeptide, which carries or directs the polypeptide through the plasma membrane of bacteria and so ensures its eventual secretion into the periplasmic space and perhaps beyond. The leader

sequence peptide is commonly removed before the polypeptide becomes active.

A single expression vector can be employed with two cistrons, for example, pComb 3, or two expression vectors can be used with one cistron each. Alternatively, an expression vector suitable for use in the present invention (for example, the SurfZap™ Vector provided in a kit by Statagene, La Jolla, California) is adapted for receiving one polynucleotide encoding both a V_H- and a V_L-encoding DNA homolog which have been directionally ligated to one another, preferably through a linker, and for expressing this polynucleotide as a single fusion protein that includes a filamentous phage coat protein membrane anchor and a prokaryotic secretion signal. The skilled artisan will appreciate that vectors suitable for expression of a heterodimeric antibody material on the surface of a filamentous phage particle can be constructed in many different ways to achieve this intended result. Thus, the skilled artisan may choose to use a combination of monocistronic expression vectors or a single expression vector with one or more cistrons.

Preferably, a phagemid expression vector for expressing heterodimeric antibody material provides a system for independently cloning (inserting) V_H- and V_L-encoding homologs into two separate expression cassettes present in the vector, to form two separate cistrons for expressing the encoded V_H and V_L polypeptides of the heterodimeric antibody material. Phagemid expression vectors comprising two expression cassettes is referred to as a dicistronic phagemid expression vector. Presently preferred dicistronic phagemid expression vectors are pComb 3 and $C_3AP313H_6$, both of which were provided by Carlos Barbas III of the Scripps Research Institute, La Jolla California. The pComb 3 phagemid will be described in detail below to provide

examples of the preferred attributes of a dicistronic phagemid expression vector.

The pComb 3 phagemid comprises a first expression cassette (also referred to herein as the "Hc2 expression cassette") that includes upstream and downstream translatable DNA sequences operatively linked via a sequence of nucleotides adapted for directional ligation to a DNA homolog. The upstream translatable sequence encodes a prokaryotic periplasmic secretion signal ("pelB leader"). The presence of the pelB leader facilitates secretion of a polypeptide fused thereto (e.g., V_H or V_L polypeptide) from the bacterial cytoplasm to the periplasmic space. Exemplary amino acid sequences of suitable pelB leader are provided in Table 1 of International Patent Application No. PCT/US93/08364, incorporated herein by reference. The downstream translatable sequence of the first expression cassette of the pComb 3 phagemid encodes the filamentous phage coat protein membrane anchor domain of the filamentous phage coat protein III. The membrane anchor domain is a portion of the carboxy terminal region of the coat protein III ("cpIII") and includes a region of the hydrophobic amino acid residues for spanning a lipid bilayer membrane, and a region of charged amino acid residues normally found at the cytoplasmic space of the membrane and extending away from the membrane. This phage coat protein membrane anchor is capable of binding the matrix of a filamentous phage particle, thereby incorporating into the phage surface a polypeptide fused thereto. Exemplary amino acid sequences of suitable filamentous phage coat protein membrane anchor domain, cpIII and cpVIII, are also provided in Table 1 of International Patent Application No. PCT/US93/08364, incorporated herein by reference.

The term "fusion protein" as used herein refers to an amino acid polymer comprised of at least two polypeptides

and a linking sequence to operatively link the two polypeptide into one continuous polypeptide. The two polypeptides linked in a fusion protein are typically derived from two independent sources, and therefore a fusion protein comprises two linked polypeptides not normally found linked in nature.

This first expression cassette of the pComb 3 phagemid includes DNA expression control sequences for expressing translatable DNA sequences. DNA expression control sequences comprise a set of DNA expression signals for expressing a structural gene product and include both 5' and 3' transcriptional promoter and terminator elements, as is well known, operatively linked to the remainder of the expression cassette such that the expression cassette is able to express a structural gene product. The set of nucleotides defining DNA expression control sequences, the upstream and downstream translatable DNA sequences and the sequence of nucleotides adapted for directional ligation to a DNA homolog are collectively referred to as an expression cassette. The 5' control sequences define a promoter for initiating transcription (transcriptional promoter) and a ribosome binding site operatively linked at the 5' terminus of the upstream translatable DNA sequence. The 3' control sequences define at least one termination (stop) codon in frame with and operatively linked to the sequence encoding the membrane anchor polypeptide.

The pComb 3 phagemid also contains a second expression cassette (also referred to herein as the "Lc2 expression cassette") for expressing a second polypeptide (e.g., either V_H or V_L polypeptide, which ever is not expressed via the first expression cassette). The second expression cassette includes a second translatable DNA sequence that encodes a pelB leader, operatively linked at its 3' terminus via a sequence of nucleotides adapted for directional ligation to

a downstream DNA sequence of the vector that typically defines at least one stop codon in the reading frame of the cassette. The second translatable DNA sequence is operatively linked at its 5' terminus to DNA expression control sequences forming the 5' elements. The second expression cassette is capable, upon insertion of a DNA sequence (e.g., a V_H- or V_L-encoding DNA homolog), of expressing the second polynucleotide encoded thereby as a fusion protein comprising the pelB leader linked with the polypeptide encoded by the inserted DNA.

A cistron in a phagemid expression vector useful in the practice of the present invention is the region of the vector that forms, upon insertion of a V_H- or V_L-encoding DNA homolog, a sequence of nucleotides capable of expressing, in an appropriate host, antibody material of UC*. Thus, the expression-competent sequence of the nucleotides is referred to as a cistron. A cistron is formed when a V_H- or V_L-encoding DNA homolog is directionally inserted ("directionally ligated") between the upstream and downstream sequences via the sequence of nucleotides adapted for that purpose. The resulting three translatable DNA sequences, namely the upstream, the inserted and the downstream sequences are all operatively linked in the same reading frame. Thus, a dicistronic phagemid expression vector for expressing heterodimeric antibody material of UC* provides a system for cloning a member of each of the V_H and V_L libraries into the cassette portions of the vector to produce cistrons capable of co-expressing a V_H and a V_L polypeptide of heterodimeric antibody material of UC*.

The pComb 3 phagemid expression vector also carries an ampicillin selectable resistance marker gene in addition to the first and second expression cassettes. An f1 phage origin of replication facilitates the generation of single-stranded phagemic. Isopropyl thiogalactopyranoside (IPTG)

induces expression of a dicistronic message encoding the fusion protein of the first cistron and the fusion protein of the second cistron.

As used herein, the term "vector" refers to a recombinant DNA molecule capable of transporting between different genetic environments another DNA molecule to which it has been operatively linked. Vector are capable of autonomous replication in a cell and to which a DNA segment, e.g., gene or polynucleotide, can be operatively linked so as to bring about replication of the attached segment. Vectors capable of directing the expression of translatable DNA and coding for one or more polypeptides are referred to herein as "expression vectors."

As used herein with regard to DNA sequences or segments, the phrase "operatively linked" means the sequences or segments have been covalently joined, preferably by a conventional phosphodiester bond, into one strand of DNA, whether in single or double-stranded form.

The choice of vector to which transcription unit or a cassette of this invention is operatively linked depends directly, as is well known in the art, on the functional properties desired, e.g., vector replication and protein expression, and the host cell to be transformed, these being limitations inherent in the art of constructing recombinant DNA molecules.

A sequence of nucleotides adapted for directional ligation, i.e., a polylinker, is a region of the phagemid expression vector that (1) operatively links for replication and transport the upstream and downstream translatable DNA sequences and (2) provides a site or a means for directional ligation of a DNA homolog into the vector. Typically, a directional polylinker is a sequence of nucleotides that defines two or more restriction endonuclease recognition sequences, or restriction sites. Under restriction

cleavage, the two sides yield cohesive termini to which a V_H- or V_L-encoding DNA homolog can be ligated to the phagemid expression vector. Preferably, the two restriction sites provide upon restriction cleavage, cohesive termini that are non-complimentary and thereby permit directional insertion of a DNA homolog into the expression cassette. For example, the sequence of nucleotides adapted for directional ligation in the first expression cassette ("Hc2") of the pComb 3 phagemid, encodes 5' to 3' the Xho 1 restriction site and the Spe I restriction site. The sequence of nucleotides adapted for directional ligation in the second expression cassette ("Lc2") of the pComb 3 phagemid encodes 5' to 3' the Sac I restriction site and the Xba I restriction site.

In a preferred embodiment, a phagemid expression vector is designed for convenient manipulation in the form of a filamentous phage particle encapsulating a genome according to the teaching of the present invention. In this embodiment, a phagemid expression vector further contains a nucleotide sequence that defines a filamentous phage origin of replication such that the vector, upon presentation of the appropriate genetic complimentation, can replicate as a filamentous phage in single-stranded replicative form and be packaged into filamentous phage particles. This feature provides the ability of the phagemid expression vector to be packaged into phage particles for subsequent segregation of the particles, and vector contained therein, away from other particles that comprise a population of phage particles.

A filamentous phage origin of replication is a region of the phage genome, as is well known, that defines sites for initiation of replication, termination of replication and packaging of the replicative form produced by replication. See, for example, Rasched et al., Microbiology Review, 50: 401-427 (1986); and Horiuchi, Journal of Molecular Biology, 188: 215-223 (1986).

A preferred filamentous phage origin of replication for use in the present invention is a M13, f1 or fd phage origin of replication. The pComb 3 phagemid employs the f1 phage origin of replication. A preferred phagemid expression vector is a dicistronic phagemid expression vector.

3. Randomly Combining V_H- and V_L-encoding DNA Homologs in a Dicistronic Phagemid Expression Vector

The construction of a library of dicistronic phagemid expression vectors capable of expressing V_H and V_L polypeptides of the V_H and V_L libraries of UC* is preferably accomplished in two general steps. In the first step, members of either the V_H or V_L libraries of UC* are directionally ligated into one of the expression cassettes of the discistronic vector. In the second step, members of the other gene library are directionally ligated into the other expression cassette, so that the dicistronic vector contains a random combination of two DNA homologs, one encoding a V_H polypeptide and the other encoding a V_L polypeptide. This results in a library of clones each of which potentially co-expresses a heavy and light chain of UC*. The actual combinations are random and do not necessarily reflect the combinations present in the B cell population in the LPL of UC patients.

In one embodiment of the present invention, a library of dicistronic phagemid expression vectors capable of expressing heterodimeric antibody material of UC* on phage particles is prepared. Each member of the dicistronic phagemid expression vector library is capable of expressing a V_H polypeptide and a V_L polypeptide from a first and a second cistron, respectively, that can form, in a suitable host, a heterodimeric antibody material of UC* on the surface of a filamentous phage particle.

In accordance with another embodiment of the present invention there is provided methods for producing a library of dicistronic phagemid expression vectors encoding heterodimeric antibody material of an immunoglobulin gene repertoire of UC*, comprising: (a) forming a first ligation admixture by combining in a ligation buffer (I) a first library of the immunoglobulin gene repertoire of UC*, said first library comprising a plurality of DNA homologs in the form of dsDNA, each DNA homolog of the library having cohesive termini adapted for directional ligation, wherein said library is selected from the group consisting of a V_H library of UC* and a V_L library of UC*, and (ii) a plurality of phagemid expression vectors in linear form, each having upstream and downstream first cohesive termini that are adapted for directionally receiving a DNA homolog of the first library of the immunoglobulin gene repertoire of UC* in a common reading frame, and wherein said first cohesive termini are operatively linked to respective upstream and downstream translatable DNA sequences, which in turn are operatively linked to respective upstream and downstream DNA expression control sequences. The upstream translatable DNA sequence of the first cohesive termini encodes a prokaryotic secretion signal and the downstream translatable DNA sequence of the first cohesive termini encodes a filamentous phage coat protein membrane anchor.

(b) subjecting the admixture to ligation conditions for a time period sufficient to operatively link DNA homologs of the first library of the immunoglobulin gene repertoire of UC* to the vectors and produce a plurality of circular phagemid expression vectors each having a first cistron for expressing the first library of the immunoglobulin gene repertoire of UC*;

(c) treating the plurality of circular phagemid expression vectors under DNA cleavage conditions to produce a plurality of phagemid expression vectors in linear form that each have upstream and downstream second cohesive termini that (I) are adapted for directionally receiving a DNA homolog of a second library of the immunoglobulin gene repertoire of UC* in a common reading frame, and (ii) are operatively linked to respective upstream and downstream translatable DNA sequences which in turn are operatively linked to DNA expression control sequences. The upstream DNA sequence of the second cohesive termini is a translatable sequence encoding a prokaryotic secretion signal and the downstream DNA sequence of the second cohesive termini has at least one stop codon in the reading frame.

(d) forming a second ligation admixture by combining in a ligation buffer (I) the plurality of phagemid expression vectors formed in (c), and (ii) the second library of the immunoglobulin gene repertoire of UC*, said second library comprising a plurality of DNA homologs in the form of dsDNA, each DNA homolog of the library having cohesive termini adapted for directional ligation to the second cohesive termini of the phagemid expression vectors, wherein said library is selected from the group consisting of a V_H library of UC* and a V_L library of UC*; and

(e) subjecting the second admixture to ligation conditions for a time period sufficient to operatively link DNA homologs of the second library of the immunoglobulin gene repertoire of UC* to said vectors and produce a plurality of

circular phagemid expression vectors each having a second cistron for expressing the second library of the immunoglobulin gene repertoire of UC* thereby forming the library of dicistronic phagemid expression vectors.

In preferred embodiments the prokaryotic secretion signal encoded by the upstream translatable DNA sequence of the first cohesive termini and the prokaryotic secretion signal encoded by the upstream translatable DNA sequence of the second cohesive termini is a pelB secretion signal. Also preferred is that the filamentous phage coat protein membrane anchor encoded by the downstream translatable DNA sequence of the first cohesive termini is derived from cp III or cp VIII as described herein.

Dicistronic phagemid expression vectors useful for practicing the above method are the dicistronic phagemid expression vectors pComb 3 and $C_2AP313H_6$.

In practicing the methods for producing a library of dicistronic phagemid expression vectors encoding heterodimeric antibody material of an immunoglobulin gene repertoire of UC*, it is preferred that the upstream and downstream first cohesive termini do not have the same nucleotide sequence as the upstream and downstream second cohesive termini. In this embodiment, treating the plurality of circular phagemid expression vectors to produce a plurality of phagemid expression vectors in linear form, typically involves the use of restriction endonucleases that are specific for producing said second cohesive termini, but do not cleave the circular phagemid expression vector at the sites that formed the first cohesive termini. Exemplary and preferred first and second termini are the termini defined by cleavage of pComb 3 with Xho I and Spe I to form the upstream and downstream first cohesive termini, and defined by cleavage of pComb 3 with Sac I and Xba I to form the

upstream and downstream second cohesive termini. In this embodiment, other pairs of cohesive termini can be utilized at the respective pairs of first and second cohesive termini, so long as the four termini are each distinct, non-complimentary termini. Exemplary are the termini found on the vectors pCBAK8, pComb2-3, pComb2-3', pComb8 and pComb2-8 described in International Patent Application No. PCT/US93/08364, incorporated herein by reference.

Methods of treating the plurality of circular phagemid expression vectors under DNA cleavage conditions to form linear phagemid expression vectors are generally well known and depend on the nucleotide sequence to be cleaved and the mechanism for cleavage. Preferred treatments involve admixing the phagemid expression vector with a restriction endonuclease specific for endonuclease recognition site at the desired cleavage location and in amount sufficient for the restriction endonuclease to cleave the phagemid expression vector. Buffers, cleavage conditions, and substrate conditions of restriction endonuclease cleavage are well known and depend on the particular enzymes utilized. Exemplary restriction enzyme cleavage conditions are described in the EXAMPLES below.

In another embodiment of the present invention, there is provided methods for producing a library of dicistronic phagemid expression vectors encoding heterodimeric antibody material from an immunoglobulin gene repertoire of UC*, comprising:

(a) forming a first ligation admixture by combining in a ligation buffer (I) a V_L-encoding DNA homolog library of UC* in the form of dsDNA, wherein each V_L-encoding DNA homolog of the library has at its 5' end a Sac I cohesive termini and at its 3' end a Xba I cohesive termini, and (ii) a plurality of pComb 3 phagemid expression vectors in linear form

each having a 5' and 3' cohesive termini adapted for directionally receiving a V_L-encoding DNA homolog of the V_L-encoding DNA homolog library of UC* in a common reading frame, wherein said 5' cohesive terminus is a Xba I cohesive terminus operatively linked to an upstream pelB leader sequence, wherein said 3' cohesive terminus is a Sac I cohesive terminus operatively linked to a downstream translatable DNA sequences having at least one stop codon in the reading frame, and wherein upstream pelB leader sequence and said downstream translatable DNA sequence are operatively linked to respective upstream and downstream DNA expression control sequences;

(b) subjecting the first ligation admixture to ligation conditions for a time period sufficient to operatively link said V_L-encoding DNA homologs to said pComb 3 vectors and produce a plurality of circular pComb 3 vectors each having a first cistron for expressing a V_L-encoding DNA homolog of the V_L-encoding DNA homolog library of UC*;

(c) treating the plurality of circular pComb 3 vectors under DNA cleavage conditions to produce a plurality of pComb 3 vectors in linear form adapted for directionally receiving a V_H-encoding DNA homolog of the V_H-encoding DNA homolog library of UC* in a common reading frame and each having (I) a Xho I cohesive terminus at its 3' end, said Xho I cohesive terminus operatively linked to an upstream translatable DNA sequence encoding a pelB leader, said upstream translatable DNA sequence operatively linked to an upstream DNA expression control sequences, and (ii) a Spe I cchesive terminus at its 5' end, said Spe I cohesive

terminus operatively linked to a downstream DNA sequence encoding a filamentous phage coat protein membrane anchor, said downstream DNA sequence operatively linked to a downstream DNA expression control sequences;

(d) forming a second ligation admixture by combining in a ligation buffer (I) the plurality of pComb 3 vectors in linear form, and (ii) a V_H-encoding DNA homolog library of UC* in the form of dsDNA adapted for directional ligation to the plurality of pComb 3 vectors, wherein each V_H-encoding DNA homolog of the library has at its 5' end a Xho I cohesive terminus and at its 3' end a Spe I cohesive terminus; and

(e) subjecting the second ligation admixture to ligation conditions for a time period sufficient to operatively link the V_H-encoding DNA homolog library to said pComb 3 vectors and produce a plurality of circular pComb 3 vectors each having the second cistron for expressing a V_H-encoding DNA homolog of the V_H-encoding DNA homolog library of UC*, thereby forming the dicistronic library.

In yet another embodiment of the present invention there is provided a library of phagemid expression vectors containing cDNA encoding V_L or V_H polypeptides from an immunoglobulin gene repertoire of UC*, wherein said immunoglobulin gene repertoire of UC* is derived from LPL of one or more humans diagnosed with UC and seropositive for pANCA. Preferably, the library of phagemid expression vectors contain cDNA encoding V_L polypeptides of the kappa isotype or V_H polypeptides of the gamma isotype. Even more preferably, the library of phagemid expression vectors contain cDNA encoding V_L polypeptides from each family of the immunoglobulin kappa light chain variable segments or V_H

polypeptides from each family of immunoglobulin heavy chain variable segments. Optionally, the cDNA encoding said V_L or V_H polypeptides is operatively linked to upstream and preferably also a downstream translatable DNA sequences, which in turn are operatively linked to respective upstream and downstream DNA expression control sequences, wherein the upstream translatable DNA sequence encodes a prokaryotic secretion signal, preferably pelB leader, and the downstream translatable DNA sequence encodes a filamentous phage coat protein membrane anchor, preferably a cpIII membrane anchor.

In yet another embodiment of the present invention there is provided a plurality of prokaryotic cells, preferably *E. coli*, containing a library of phagemid expression vectors containing cDNA encoding V_L or V_H polypeptides from an immunoglobulin gene repertoire of UC^+ of the present invention. In a related embodiment, the plurality of prokaryotic cells contain both a library of phagemid expression vectors containing cDNA encoding V_L polypeptides from an immunoglobulin gene repertoire of UC^+ and a library of phagemid expression vectors containing cDNA encoding V_H polypeptides from an immunoglobulin gene repertoire of UC^+.

An alternative embodiment provides a population of filamentous phage particles encapsulating a library of phagemid expression vectors containing cDNA encoding V_L or V_H polypeptides from an immunoglobulin gene repertoire of UC^+ of the present invention. In a related embodiment, the plurality of prokaryotic cells contain both a library of phagemid expression vectors containing cDNA encoding V_L polypeptides from an immunoglobulin gene repertoire of UC^+ and a library of phagemid expression vectors containing cDNA encoding V_H polypeptides from an immunoglobulin gene repertoire of UC^-. Preferably, the V_L or V_H polypeptides encoded by cDNA contained in the phagemid expression vector

is expressed on the surface of the phagemid particle which encapsulates it as a fusion protein comprising a V_L or V_H polypeptide and a filamentous phage coat protein membrane anchor.

Another library of the present invention is embodied in a library of heterodimeric phagemid expression vectors encoding and capable of expressing heterodimeric antibody material from an immunoglobulin gene repertoire of UC*, wherein said immunoglobulin gene repertoire of UC* is derived from LPL of one or more humans diagnosed with UC and seropositive for pANCA. One cistron of the dicistronic phagemid expression vectors contains cDNA encoding V_L polypeptides and the other cistron contains cDNA encoding V_H polypeptides. Preferably, the V_L-encoded polypeptides are of the kappa isotype and, even more preferably, each family of immunoglobulin kappa light chain variable segments is represented in the V_L-encoded polypeptides. It is also preferred that each of the immunoglobulin heavy chain variable segment families is represented in the V_H-encoded polypeptides, and that the V_H-encoded polypeptides are of the gamma isotype. Both the cDNA encoding said V_L polypeptides and the cDNA encoding said V_H polypeptides are operatively linked to an upstream translatable DNA sequence encoding a prokaryotic secretion signal, preferably pelB leader, which in turn is operatively linked to an upstream DNA expression control sequence. Either the cDNA encoding said V_L polypeptides or V_H polypeptides is operatively linked to a downstream translatable DNA sequences encoding a filamentous phage coat protein membrane anchor, preferably a cpIII membrane anchor, which in turn is operatively linked to a downstream DNA expression control sequences. Preferably, the dicistronic phagemid expression vectors is pComb 3 or $C_3AP313H_6$.

In one embodiment, the libraries of heterodimeric phagemid expression vectors of the present invention may be contained in a population of prokaryotic cells, for example, a population of *E. coli*. An embodiment of a library of heterodimeric phagemid expression vectors encoding and capable of expressing heterodimeric antibody material from an immunoglobulin gene repertoire of UC*, which was produced in accordance with the methods of the present invention and are contained in a population of *E. coli* cells, has been deposited with the American Type Culture Collection ("ATCC"), 12301 Parklawn Drive, Rockville, Maryland, U.S.A. 20852, on May 31, 1995 and assigned ATCC Accession No. 69827 under the terms of the Budapest Treaty on the International Recognition of Deposits of Microorganisms for Purposes of Patent Procedure and the Regulations promulgated under this Treaty. Samples of the deposited material are and will be available to industrial property offices and other persons legally entitled to receive them under the terms of the Treaty and Regulations and otherwise in compliance with the patent laws and regulations of the United States of America and all other nations or international organizations in which this application, or an application claiming priority of this application, is filed or in which any patent granted on any such application is granted. In particular, upon issuance of a U.S. patent based on this or any application claiming priority to or incorporating this application by reference thereto, all restriction upon availability of the deposited material will be irrevocably removed.

F. Surface-Expression of Antibody Material of UC+ on Filamentous Phage That Encapsulate Expression Vector

1. Filamentous Phage

Filamentous bacteriophages are a group of related viruses that infect bacteria. They are termed filamentous because they are long, thin particles comprised of a proteineous shell or coat that encases the phage DNA. The F pili filamentous bacteriophage ("Ff phage") infect only gram-negative bacteria by specifically adsorbing to the tip of the F pili of the bacteria, and include Fd, F1 and M13. The Ff phage neither kill the host cell, nor cause lysis.

The coat of mature Ff phage is comprised of five proteins encoded by the phage DNA. The length of the phage coat is formed by 2500 to 3000 copies of coat protein VIII ("cpVIII") in an ordered helix array that forms the characteristic filament structure. About five copies each of the other four coat proteins are present at the ends of the elongated coat: cpIII and cpIV at one end of the coat, and cpVII and cpIX at the other. The cpIII, encoded by gene III of the phage DNA, serves as a receptor for binding of the phage to its bacterial host in the initial phase of infection. For detailed reviews of Ff phage structure, see Rasched et al., Microbiology Review, 50: 401-427 (1986); and Model et al., in "The Bacteriophages, Volume 2," Our Calendar, et. Plenum Press, pp. 375-456 (1988), all of which are incorporated herein by reference.

The assembly of an Ff phage particle involves highly complex mechanics. Generally, though, the phage particles are assembled during extrusion of the viral genome through the host cell's membrane. Prior to extrusion, cpVIII and cpIII are synthesized and transported to the host cell's membrane. Both cpVIII and cpIII are anchored in the host

cell membrane prior to their incorporation into the mature particle.

Both cpIII and cpVIII proteins include two domains that provide signals for assembly of the mature phage particle. The first domain is a secretion signal that directs the newly synthesized protein to the host cell membrane. The secretion signal is located at the amino terminus of the polypeptide and targets the polypeptide at least to the cell membrane. The second domain is a membrane anchor domain that provides signals for association with the host cell membrane and for association with the phage particle during assembly. This second signal for both cpIII and cpVIII comprises at least a hydrophobic region for spanning the membrane.

It has been shown through manipulating the sequence of cpIII that the C-terminal 23 amino acid residue stretch of hydrophobic amino acids normally responsible for a membrane anchor function can be altered in a variety of ways and retain the capacity to associate with the membranes. Ff phage-based expression vectors have been described in which the entire cpIII amino acid residue sequence was modified by insertion of a short polypeptide or an amino acid residue sequence defining a single chain antibody domain. See, Parmley et al., Gene, 73: 305-318 (1988); Cwirla et al., Proceedings of the National Academy of Science, USA, 87: 6378-6382 (1990); and McCafferty et al., Science, 348: 552-554 (1990), incorporated by reference herein. These hybrid proteins were synthesized and assembled into phage particles in amounts of about five copies per particle, a density at which normal cpIII is usually found. In addition, enzymatically functional alkaline phosphatase has been expressed on the surface of filamentous phage particles as a fusion protein with cpIII. McCafferty et al., Protein Engineering, 4: 955-961 (1991).

2. Methods for Producing Filamentous Phage

A filamentous phage particle of this invention is produced by standard filamentous phage particle preparation methods and depends on the presence of a phagemid expression vector of this invention containing a filamentous phage origin of replication as described herein to provide the signals necessary for (1) production of a single-stranded filamentous phage replicative form and (2) packaging of the replicative form into a filamentous phage particle. Such a phagemid expression vector can be packaged when present in a bacterial cell host upon introduction of genetic complimentation to provide the filamentous phage proteins required for production of infectious phage particles.

Generally, the method for producing filamentous phage particles having on the particle surface heterodimeric antibody material of UC* comprises: (a) introducing into a prokaryotic host cell permissive for filamentous phage replication a dicistronic phagemid expression vector containing and capable of expressing V_H-encoding and V_L-encoding DNA homologs of UC*, wherein one of the encoded polypeptides is fused to a filamentous phage coat protein membrane anchor, and b) maintaining the prokaryotic host cell containing the vector under conditions sufficient for filamentous phage production and under conditions sufficient for expression of the heterodimeric antibody material of UC*, thereby forming the phage particle.

Introducing a dicistronic phagemid expression vector into a permissive prokaryotic host is accomplished by transformation, for example, of *E. coli*, with the vector. Transformation of a prokaryotic host cell is well known and includes calcium-mediated transformation, electroporation and the like. Other introducing means include infection by a filamentous phage particle.

A prokaryotic host cell useful for producing a filamentous phage of this invention is one permissive for filamentous infection and morphogenesis, and is well characterized in the filamentous phage arts. A preferred host is an *E. coli* cell, although other prokaryotic cells may be used.

Maintaining in accordance with (b) above is conducted to facilitate expression and assembly of the DNA homologs in the introduced vector to form the phage particle. Typically, a phagemid expression vector of this invention contains the minimum genetic information for the preparation and manipulation of recombinant DNA molecules, and as such, does not contain the complete range of genes required for production of a filamentous phage particle. A typical and preferred method for genetic complimentation is to infect a bacterial host cell containing a phagemid expression vector of this invention with a helper filamentous phage, thereby providing the genetic element required for phage particle assembly. Exemplary helper rescue methods are described herein in the EXAMPLES, and described by Short et al., Nucleic Acid Research, 16: 7583-7600 (1988), incorporated herein by reference.

Thus, the maintaining step typically includes a superinfection by helper phage combined with an incubation period under conditions for allowing the helper genome to express the complimenting genes and to assist the expression and assembly of a phage particle.

The amount of heterodimeric antibody material of UC* captured on the surface of filamentous phage particle during the process of phage particle extrusion from the host cell can be controlled by a variety of means. In one embodiment, the amount of heterodimeric antibody material of UC* on the phage particle surface can be controlled by controlling the timing between expression of the V_H and V_L fusion proteins

and the superinfection by helper phage. After introduction of the expression vector into the host cell, longer delay times before the addition of helper phage will allow for increased accumulation of the fusion proteins in the host cell, thereby increasing the amount of fusion protein captured by the extruding phage particle.

Thus, in accordance with a preferred embodiment of the present invention, a library of pComb 3 expression vectors encoding heterodimeric antibody material from the immunoglobulin gene repertoire of UC* is introduced into *E. coli* by transformation. One cistron of each pComb 3 expression vector contains cDNA encoding a V_L polypeptide and the other cistron of each vector contains cDNA encoding a V_H polypeptide. Both the cDNA encoding said V_L polypeptides and the cDNA encoding said V_H polypeptides are operatively linked to an upstream translatable DNA sequence encoding a prokaryotic secretion signal, preferably pelB leader, which in turn is operatively linked to an upstream DNA expression control sequence. Either the cDNA encoding said V_L polypeptides or V_H polypeptides is operatively linked to a downstream translatable DNA sequences encoding a filamentous phage coat protein membrane anchor, preferably a cpIII membrane anchor, which in turn is operatively linked to a downstream DNA expression control sequences. The f1 phage origin of replication in pComb 3 facilitates the generation of single-stranded phagemid. Isopropyl thiogalactopyranoside (IPTG) induces expression of the dicistronic message. The prokaryotic secretion signal, e.g., pelB leader, which is subsequently cleaved, facilitates the coordinated but separate secretion of both the V_L and V_H polypeptides from the bacterial cytoplasm to the periplasmic space. If, for example, the V_H polypeptide is fused to cpIII, it will become anchored in the membrane via the cpIII membrane anchor while the V_L polypeptide is

secreted into the periplasm. The V_H polypeptide in the presence of the V_L polypeptide assembles to form the heterodimeric antibody material of UC*, preferably Fab molecules. The same result can be achieved if, in the alternative, the V_L polypeptide is anchored in the membrane via a V_L polypeptide/membrane anchor fusion protein and a soluble V_H polypeptide is secreted via a pelB leader into the periplasm. With the subsequent infection of *E. coli* with helper phage as the assembly of the filamentous bacteriophage progresses, cpIII is incorporated on the tail of the bacteriophage anchoring the antibody material of UC* to the surface of the bacteriophage.

Thus, the present invention provides a population of filamentous phage particles which encapsulate a library of dicistronic phagemid expression vectors encoding heterodimeric antibody material from the immunoglobulin gene repertoire of UC* which can be produced in accordance with the methods of the present invention. In a related embodiment members of the population of filamentous phage also have expressed on the surface of the filamentous phage the heterodimeric antibody material encoded by the expression vector it encapsulates.

G. Segregation of UCpANCA-Expressing Phage Particles from a Phage Library

Where a library of the present invention is produced by first separately cloning V_H and V_L gene repertoires of UC* LPL, corresponding to the heavy and light chain polypeptides of the heterodimeric antibody material of UC*, the resulting library size after randomly combining the two repertoires in the form of a dicistronic vector is greatly increased. For example, consider light chain and heavy chain variable antibody gene repertoires, each having 10^6 different members. Combining the two repertoires theoretically yields

a phage library containing 10^{12} possible different heterodimeric antibody material species.

Isolation (segregation) of a phage particle containing a DNA expression vector encoding the V_H and/or V_L polypeptides of UCpANCA material is typically conducted by segregation of the filamentous phage particle containing the DNA homolog(s) of interest away from the population of other phage particles comprising the library. Segregation of phage particles involves the physical separation and propagation of individual phage particles away from other particles in the library. Methods for physical separation of filamentous phage particles to produce individual particles, and the propagation of the individual particles to form populations of progeny phage derived from the individual segregated particle are generally well known in the filamentous phage arts.

A preferred separation method involves the identification of the expressed heterodimeric UCpANCA material on the surface of a phage particle by means of a UCpANCA antigen binding specificity between the phage particle and UCpANCA antigen. Exemplary and preferred is the use of "panning" methods whereby a suspension of phage particles is contacted with a solid phase antigen, for example methanol fixed neutrophil, and allowed to immunoreact. After binding, non-bound particles are washed off the solid phase, and the bound phage particles are those that contain UCpANCA antigen-specific immunoglobulin polypeptides on their surface. The bound particles can then be recovered by elution of the bound particle from the solid phase, typically by the use of aqueous solvents that interfere with the antigen-antibody material interaction. Typical solvent include buffers having high ionic strength or low pH.

An alternative method for separating a phage particle based on the UCpANCA antigen specificity of the surface-expressed heterodimer antibody material of UC* from a population of particles is to precipitate the phage particles from the solution phase by cross linkage with UCpANCA antigen, for example methanol fixed neutrophil.

The use of the above particle segregation methods provides a means for screening a population of filamentous phage particles present in a phage library of this invention. As applies to a phage library, screening can be utilized to enrich the library for one or more particles that express UCpANCA material having specificity for UCpANCA antigen. Where the library is designed to contain multiple species of UCpANCA material that all have some detectable measure of UCpANCA antigen binding activity, but differ in protein structure, antigenicity, antigen binding affinity or avidity, and the like, the screening methods can be utilized sequentially to first produce a library enriched for a preselected binding specificity, and then to produce a second library further enriched by further screening comprising one or more isolated phage particles. Methods for measuring antigen binding activities, antigenicity and the like interactions between an antigen and antibody material are generally well known and are not discussed further as they are not essential features of the present invention.

Thus, the present invention provides a population of filamentous phage particles encapsulating dicistronic phagemid expression vectors encoding heterodimeric antibody material of UC*, wherein said heterodimeric antibody material immunoreacts with UCpANCA antigen as demonstrated by binding to methanol fixed neutrophil. Accordingly, said heterodimeric antibody material is referred to herein as UCpANCA antibody material. In another embodiment, the

present invention a population of filamentous phage particles that are the progeny of a single particle, and therefore all express the same UCpANCA material on the particle surface. Such a population of phage are homogeneous and clonally derived, and therefore provide a source for expressing large quantities of the UCpANCA material.

H. Production of Soluble Antibody Material from Phagemid Encoding Antibody Material

Heterodimeric antibody material of UC* anchored to the coat of a phage particle, in accordance with the present invention, can be expressed in a soluble form simply by excising the polynucleotide encoding the phage coat protein membrane anchor domain from the discistronic phagemid expression vector encoding the heterodimeric antibody material. Thus, the V_H- and V_L-encoding DNA homologs will be expressed as a pelB/V_H fusion protein and a pelB/V_L fusion protein, respectively. Each will be secreted into the periplasmic space and will assemble into heterodimer antibody material of UC*, but will not be anchored. Such soluble antibody material can be recovered from the host cell extract for subsequent use or further characterization.

For example, the pComb 3 expression vector contain cDNA encoding V_H and V_L polypeptides can be isolated and digested with Spe I and Nhe I restriction endonucleases to excise the polynucleotide encoding the cpIII anchor domain. Because Spe I and Nhe I produce compatible cohesive ends, the vector can be gel-purified, for example, and self-ligated yielding a phagemid that can be induced to express soluble antibody material in a transformed host.

I. UCpANCA Material

Soluble heterodimeric UCpANCA material generated as described above is amenable to further characterization in accordance with one or more of the immunometric assays also described herein. Thus, in accordance with the present invention, there is provided heterodimeric UCpANCA material having immunoreactivity with a nuclear antigen in neutrophils, wherein said immunoreactivity is characterized by a perinuclear staining pattern generated in an alcohol-fixed neutrophil IIF assay, as described in the EXAMPLES below. Preferably, the immunoreactivity of said UCpANCA material is further characterized as being disrupted by pre-treatment of alcohol-fixed neutrophil with DNase, as can be demonstrated, for example, by the DNase sensitivity assay described in the EXAMPLES. Disruption of the immunoreactivity of UCpANCA can display itself in the IIF assay as a loss of the pANCA staining pattern, or a change in staining pattern from a pANCA staining pattern to a cANCA staining pattern. Even more preferably, the immunoreactivity of said UCpANCA material is further characterized as being localized within the nuclear envelop of the neutrophil, as can be demonstrated, for example by confocal microscopy or immune electron microscopy as described herein. Still more preferably, said UCpANCA material is a Fab having a molecular weight of about 60,000 Daltons on 12% SDS-PAGE.

The present invention also provides a dicistronic phagemid expression vectors encoding the heterodimeric UCpANCA material of the present invention.

Since the DNA homologs encoding the V_H and V_L polypeptides of UCpANCA can be produced in large quantities, excised from the dicistronic phagemid expression vectors, and sequences by methods well known in the art, the UCpANCA material produced in accordance with the present invention

can be defined in terms of the nucleic acid sequences and deduced amino acid sequences which encode the constituent polypeptides. As described in greater detail in the EXAMPLES, when phagemids encoding ten separate clones of UCpANCA material were digested with BstN1 and analyzed by agarose gel electrophoresis, two consistent restriction patterns were detected.

Clones representative of these two patterns (5-3 and 5-4) were directly analyzed by DNA sequencing. The nucleic acid sequences of the V_H polypeptides of clones 5-3 and 5-4 are provided in SEQ ID NO: 1 and 3, respectively. The nucleic acid sequences of the V_L polypeptides of clones 5-3 and 5-4 are provided in SEQ ID NO: 5 and 7, respectively. V, D, and J segment sequences were separately analyzed for homology to previously reported germline and re-arranged Ig genes using standard computer methodology. Figure 3 provides amino acid sequence comparison between clones 5-3, 5-4 and their closest germline counterpart. Clone 5-3 uses J_H3 with three nucleotide and two amino acid substitutions, and $J_\kappa1$ with two nucleotide substitutions. Clone 5-4 uses J_H6 with one silent nucleotide substitution and $J_\kappa3$ with two nucleotide substitutions resulting in a change to one amino acid. The diversity segment utilized by clones 5-3 and 5-4 were not assigned. Despite these differences in the use of joining segments, the clones show a high degree of homology in the heavy chain and light chain variable segments. Clone 5-3 and 5-4 both use a V_κ segment assigned to VA27, with 14 and nine amino acid substitutions, respectively. Clone 5-3 and 5-4 both also use a V_H segment assigned to DP49 (1.9III gene homolog) with 12 amino acid substitutions each. The novel shared sequence in V_H CDR2, particularly the highly charged RKKK segment contained therein, and the high replacement:silent ratio in this segment (see Table 5), indicates that it is important to antigen recognition.

Table 5. Mutation pattern in UC pANCA clones. DNA sequences from the 5-3 and 5-4 clones were compared to the putative heavy and light chain germline genes, and analyzed for the frequency of mutations resulting in replacement and silent coding changes. The replacement:silent ratio was calculated for framework (FR1-3) and complementarity-determining (CDR1-2) regions. Evidence of antigenic selection (ratio greater than ~3) is observed for the heavy and light chain CDR of both clones.

	Framework	CDR
Heavy Chain		
5-3	1.5	4
5-4	1.7	4
Light Chain		
5-3	1.3	7
5-4	3	4/0

Accordingly, the skilled artisan will appreciate that the exemplary sequence information on UCpANCA material and UCpANCA polypeptides provided herein, especially as presented in SEQ ID NOs. 1 through 8, can be re-engineered by methods well known to the skilled artisan, for example by such techniques as CDR grafting, point mutagenesis, and the like so as to generate other UCpANCA material and UCpANCA polypeptides without departing from the basic and novel features of the invention as described herein. Thus, new UCpANCA material and UCpANCA polypeptides can be generated having different sequences than the sequences specifically given in the SEQUENCE ID LISTING without destroying, and perhaps even improving, the immunoreactive characteristics of UCpANCA material and polypeptides.

For example, the present invention provides UCpANCA material and the polypeptides of UCpANCA material defined as follows with reference to SEQ ID NOs. 1 through 8. One of

skill in the art will appreciate that having provided the sequences of UCpANCA antibody material, polypeptides, and polynucleotides of the present invention, additional embodiments of such compositions can be generated which have amino acid residue sequence substantially identical to a sequence specifically shown herein merely by making conservative substitutions in one or more residues of the sequence with a functionally similar residue and which displays the ability to mimic the compositions as described herein. Examples of conservative substitutions include the substitution of one non-polar (hydrophobic) residue such as isoleucine, valine, leucine or methionine for another, the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, between glycine and serine, the substitution of one basic residue such as lysine, arginine or histidine for another, or the substitution of one acidic residue, such as aspartic acid or glutamic acid for another.

The phrase "conservative substitution" also includes the use of a chemically derivatized residue in place of a non-derivatized residue provided that such polypeptide displays the requisite binding activity.

"Chemical derivative" refers to a subject polypeptide having one or more residues chemically derivatized by reaction of a functional side group. Such derivatized molecules include, for example, those molecules in which free amino groups have been derivatized to form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups may be derivatized to form O-acyl or O-alkyl derivatives. The imidazole nitrogen of histidine may be derivatized to form

N-im-benzylhistidine. Also included as chemical derivatives are those peptides which contain one or more naturally occurring amino acid derivatives of the twenty standard amino acids. For examples: 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be substituted for serine; and ornithine may be substituted for lysine. Polypeptides of the present invention also include any polypeptide having one or more additions and/or deletions of residues relative to the sequence of a polypeptide whose sequence is shown herein, so long as the requisite activity is maintained.

There is provided isolated and/or recombinant UCpANCA polypeptides comprising at least one variable segment of an immunoglobulin heavy chain ("V_H segment"), wherein the V_H segment comprises framework regions, complementarity determining region I ("CDR_H I") and complementarity determining region II ("CDR_H II"), and wherein the CDR_H I has substantially the same amino acid sequence as residues 33 through 37 of SEQ ID NO: 2 or residues 32 through 36 of SEQ ID NO: 4, and/or wherein the CDR_H II has substantially the same amino acid sequence as residues 52 through 68 of SEQ ID NO: 2 or residues 51 through 67 of SEQ ID NO: 4, and/or wherein the V_H segment has substantially the same amino acid sequence as residues 6 through 100 of SEQ ID NO: 2 or residues 6 through 99 of SEQ ID NO: 4. More preferably, the CDR_H I has the same amino acid sequence as residues 33 through 37 of SEQ ID NO: 2 or residues 32 through 36 of SEQ ID NO: 4, and/or the CDR_H II has the same amino acid sequence as residues 52 through 68 of SEQ ID NO: 2 or residues 51 through 67 of SEQ ID NO: 4, and/or the V_H has the same amino acid sequence as residues 1 through 95 of SEQ ID NO: 2 or residues 6 through 99 of SEQ ID NO: 4.

In yet another embodiment of these polypeptides of UCpANCA material there is provided polypeptides, that further comprise an immunoglobulin heavy chain joining segment ("J_H segment") and a diversity segment, wherein at least a portion of the J_H segment and a portion of the diversity segment define a complementarity determining region III ("CDR_H III"), and wherein the amino acid sequence of the CDR_H III is substantially the same, or more preferably, the same as the amino acid sequence as residues 101 through 109 of SEQ ID NO: 2 or residues 100 through 120 of SEQ ID NO: 4.

In another embodiment of the invention, there is provided isolated, substantially pure, and/or recombinant immunoglobulin heavy chain polypeptides having substantially the same amino acid sequence as SEQ ID NO: 2 or SEQ ID NO: 4, or more preferably the same amino acid sequence as SEQ ID NO: 2 or SEQ ID NO: 4.

Any one of these polypeptides containing regions of immunoglobulin heavy chain segments can be part of, for example, an Fd polypeptide, an immunoglobulin heavy chain of a Fab, Fab', $F(ab')_2$, an antibody and the like.

There is also provided isolated, substantially pure and/or recombinant polypeptides comprising at least one variable segment of an immunoglobulin kappa light chain ("V_K segment"), wherein the V_K segment comprises framework regions, complementarity determining region I ("CDR_K I") and complementarity determining region II ("CDR_K II"), and wherein the CDR_K I has substantially the same amino acid sequence as residues 23 through 34 of SEQ ID NO: 6 or SEQ ID NO: 8, and/or wherein the CDR_K II has substantially the same amino acid sequence as residues 50 through 56 of SEQ ID NO: 6 or SEQ ID NO: 8. More preferably, the CDR_K I has the same amino acid sequence as residues 23 through 34 of SEQ ID NO: 6 or SEQ ID NO: 8, and/or wherein the CDR_K II has the

same amino acid sequence as residues 50 through 56 of SEQ ID NO: 6 or SEQ ID NO: 8.

In yet another embodiment of the isolated, substantially pure, and/or recombinant polypeptides of UCpANCA material there is provided polypeptides, that further comprise an immunoglobulin kappa light chain joining segment ("J_K segment"), wherein at least a portion of the J_H segment defines a complementarity determining region III ("CDR_K III"), and wherein the amino acid sequence of the CDR_K III is substantially the same amino acid sequence as residues 89 through 97 of SEQ ID NO: 6 or residues 89 through 98 of SEQ ID NO: 8, or more preferably, the CDR_k III has the same amino acid sequence as residues 89 through 97 of SEQ ID NO: 6 or residues 89 through 98 of SEQ ID NO: 8.

In another embodiment of the invention, there is provided isolated, substantially pure, and/or recombinant immunoglobulin kappa light chain polypeptides having substantially the same amino acid sequence as SEQ ID NO: 6 or SEQ ID NO: 8, or more preferably the same amino acid sequence as SEQ ID NO: 6 or SEQ ID NO: 8.

Any one of these polypeptides containing regions of immunoglobulin light chain segments can be part of, for example, an immunoglobulin light chain, an immunoglobulin light chain of a Fab, Fab', $F(ab')_2$, an antibody, and the like. Preferably the these polypeptides containing regions of immunoglobulin light chain segments are combined in dimeric antibody material, preferably with polypeptides of the present invention that contain regions of immunoglobulin heavy chain segments. Even more preferred is that the UCpANCA polypeptides either alone or in combination with other polypeptides, retain at least in some part the immunoreactivity of UCpANCA material of the present invention. Accordingly, the present invention provides UCpANCA polypeptides characterized as immunoreactive with

nuclear antigen of neutrophil, wherein said immunoreactivity is characterized by a perinuclear staining pattern generated in an alcohol-fixed neutrophil IIF assay, wherein said immunoreactivity is characterized as being disrupted by pre-treatment of alcohol-fixed neutrophil with DNase, and/or wherein said immunoreactivity is characterized as localized within the nuclear envelop of the neutrophil.

Also provided by the present invention are nucleic acids in the form of single stranded and double stranded cDNA ~~or~~ RNA encoding the polypeptides and antibody materials of the present invention. These nucleic acids can be incorporated into vectors. A presently preferred vector of the present invention is a dicistronic phagemid expression vector, such as for example, pComb 3. Additional vectors useful herein are viruses, such as baculoviruses and retroviruses, cosmids, plasmids, and the like. Nucleic acid molecules are inserted into vector genomes by methods well known in the art. For example, insert and vector DNA can both be exposed to a restriction enzyme to create complementary ends on both molecules that base pair with each other and which are then joined together with a ligase. Alternatively, synthetic nucleic acid linkers that correspond to a restriction site in the vector DNA, can be ligated to the insert DNA which is then digested with a restriction enzyme that recognizes a particular nucleotide sequence. Additionally, an oligonucleotide containing a termination codon and an appropriate restriction site can be ligated for insertion into a vector containing, for example, some or all of the following: a selectable marker gene, such as neomycin gene for selection of stable or transient transfectants in mammalian cells; enhancer/promoter sequences from the immediate early gene of human CMV for high levels of transcription; transcription termination and RNA processing signals from SV40 for mRNA stability; SV40

polyoma origins of replication and ColE1 for proper episomal replication; versatile multiple cloning sites; and T7 and SP6 RNA promoters for *in vitro* transcription of sense and antisense RNA. Other means are available and can readily be accessed by those of skill in the art.

Also provided are expression vectors comprising a cDNA molecule encoding UCpANCA polypeptide or antibody material, adapted for expression in a bacterial cell, a yeast cell, a mammalian cell and other animal cells. The vectors additionally comprise the regulatory elements necessary for expression of the DNA in the bacterial, yeast, mammalian or animal cells so located relative to the DNA encoding the UCpANCA polypeptide as to permit expression thereof. Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector includes a promoter such as the lac promoter and for transcription initiation, the Shine-Dalgarno sequence and the start codon AUG (Ausubel et al., *supra* 1993). Similarly a eukaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors can be obtained commercially or assembled by the sequences described in methods well known in the art, for example, the methods described above for constructing vectors in general. Expression vectors are useful to produce cells that express the polypeptide.

This invention also provides a phagemid expression vector containing cDNA encoding UCpANCA V_L or V_H polypeptides, preferably both, i.e., UCpANCA material. Optionally, the cDNA encoding said V_L or V_H polypeptide is operatively linked to upstream and preferably also a downstream translatable DNA sequences, which in turn are

operatively linked to respective upstream and downstream DNA expression control sequences, wherein the upstream translatable DNA sequence encodes a prokaryotic secretion signal, preferably pelB leader, and the downstream translatable DNA sequence encodes a filamentous phage coat protein membrane anchor, preferably a cpIII membrane anchor. Still more preferably, the phagemid expression vector is pComb 3.

In yet another embodiment of the present invention there is provided a prokaryotic cells, preferably *E. coli*, containing a phagemid expression vector which contains cDNA encoding UCpANCA V_L or V_H polypeptides, preferably both, i.e., UCpANCA material.

This invention also provides a mammalian cell containing cDNA encoding a UCpANCA polypeptide or antibody material. An example is a mammalian cell comprising a plasmid adapted for expression in a mammalian cell. The plasmid contains cDNA encoding a UCpANCA polypeptide and the regulatory elements necessary for expression of the polypeptide. Various mammalian cells may be utilized as hosts, including for example, mouse fibroblast cell NIH3T3, CHO cells, HeLa cells, Ltk- cells, etc. Expression plasmids such as those described *supra* can be used to transfect mammalian cells by methods well known in the art, for example, calcium phosphate precipitation, DEAE-dextran, electroporation, microinjection, lipofection, and the like.

The present invention further provides a filamentous phage particle comprising a proteineous filamentous phage coat including UCpANCA material, said UCpANCA material being comprised of at least one UCpANCA V_H or V_L polypeptide integrated into the surface of said phage coat via a filamentous phage coat protein membrane anchor domain that is fused to at least one of the polypeptides of the UCpANCA material. Preferably, the phage coat is encapsulating a

genome encoding the polypeptides which form the UCpANCA material.

A preferred filamentous phage of this invention comprises heterodimeric UCpANCA material comprising a UCpANCA V_L polypeptide, and a UCpANCA V_H polypeptide fused to a filamentous phage coat protein membrane anchor, forming a UCpANCA V_H fusion protein, wherein the membrane anchor portion of the UCpANCA V_H fusion protein is integrated into the phage coat and the V_H polypeptide portion of the UCpANCA V_H fusion protein binds with the UCpANCA V_L polypeptide that is otherwise a free, soluble monomer. Stated differently, the V_H polypeptide portion of the UCpANCA V_H fusion protein and the UCpANCA V_L are capable of autogenous assembly into a functional, heterodimeric UCpANCA material, which is expressed on the outer surface of the phage in a manner accessible to UCpANCA antigen, i.e., they are surface-integrated into the phage.

By soluble, what is meant is non-anchored, unattached, free, non-fusion protein, non-fusion polypeptide, non-fastened, releasable from an anchored state by treatment of a dimer having intersubunit bonds, such as disulfide bonds between two cystine residues, and the like. Therefore, the term soluble defines a heterologous polypeptide that is expressed from a vector of this invention without a membrane anchor that is free to bind to another soluble monomer or to an anchored monomer. In addition, the term soluble also defines a heterologous polypeptide that is released from a dimer by exposure of that dimer to a reducing agent, such as beta-mercaptoethanol, that results in a separation of the monomeric subunits.

The surface integration of the a heterodimeric UCpANCA material is provided by the presence of filamentous phage coat protein membrane anchor domain fused thereto. Preferably, a coat protein is selected from the group

consisting of cpIII and cpVIII. In a preferred embodiment described herein, the filamentous phage coat protein membrane anchor is cpIII. However, where cpVIII is used, the majority of the phage coated is covered with the heterodimeric UCpANCA material. Where cpIII is used as the membrane anchor the heterodimeric UCpANCA material is localized at one terminus of the phage particle.

J. Methods for Using UCpANCA Material

1. Diagnostic Systems

The present invention also describes a diagnostic system, preferably in kit form, for assaying for the presence of serum UCpANCA in humans, and thereby aiding physicians with the diagnosis of UC. Large volumes of monoclonal UCpANCA material can be produced in accordance with the methods described above which by their very nature are particularly well suited for use as a reference reagent in immunodiagnostic assays and kits for diagnosing UC. For example, the alcohol-fixed neutrophil IIF assay is a conventional assay for detecting the presence of pANCA in serum of a patient suspected of having UC. Use of UCpANCA material of the present invention as a reference reagent would provide a reliable positive control for the pANCA staining pattern associated with UC. Likewise, confocal microscopy provides a method detecting the presence of serum pANCA, thereby indicating UC. UCpANCA material can be used as a reference reagent to provide a reliable positive control to confirm localization of the immune complex to the interior of the nucleus of neutrophil.

A suitable kit of the present invention includes, for example, in an amount sufficient for at least one assay, UCpANCA material, preferably monoclonal UCpANCA of an IgG isotype as a separately packaged reagent. Preferably, the kits also include any one or more of the following in an

amount sufficient for at least one assay: neutrophil, DNase, DNase treated neutrophil, detectable marker, enzyme substrate, anti-IgG, and the like. In addition, other components such as ancillary reagents may be included, for example, stabilizers, buffers, fixatives, and the like.

"Instructions for use" typically include a tangible expression describing the reagent concentration or at least one assay method parameter such as the relative amounts of reagent and sample to be admixed, maintenance time periods for reagent/sample admixtures, temperature, buffer conditions and the like.

The neutrophil, for example, can be affixed to a solid matrix to form a solid support that comprises a package in the subject diagnostic systems. A reagent is typically affixed to a solid matrix by adsorption from an aqueous medium. Useful solid matrices are also well known in the art.

The UCpANCA material, neutrophil, labeled specific binding agent, DNase and the like of any kit described herein can be provided in solution, as a liquid dispersion or as a substantially dry power, e.g., in lyophilized form. Where the indicating means is an enzyme, the enzyme's substrate can also be provided in a separate package of a system.

The packaging materials discussed herein in relation to the kits are those customarily utilized in kits and commercially available. The term "package" refers to a solid matrix or material such as glass, plastic (e.g., polyethylene, polypropylene and polycarbonate), paper, foil and the like capable of holding within fixed limits a diagnostic reagent such a protein, polypeptide fragment, antibody or monoclonal antibody of the present invention. Thus, for example, a package can be a bottle, vial, plastic and plastic-foil laminated envelope or the like container

used to contain a contemplated reagent or it can be a microtiter plate well to which microgram quantities of a contemplated reagents have been operatively affixed, i.e., linked so as to be capable of being immunologically bound by an antibody or polypeptide to be detected.

2. Isolation and Characterization of UCpANCA Antigen

The UCpANCA material of the present invention is also well suited for the isolation characterization and cloning of UCpANCA antigen(s). Accordingly, the present invention provides methods of isolating such antigen comprising contacting UCpANCA material with neutrophil cell lysate for a time and at a temperature and pH suitable to form an immune complex comprising UCpANCA material, then separating said immune complex from non-complexed cell lysate, and separating said UCpANCA material from said antigen.

In a presently prefered embodiment, the 5-3 recombinant UCpANCA Fab clone is utilized to characterize, isolate and clone the UCpANCA reactive antigen by immunoaffinity purification. This technique is one of the most powerful methods for isolation of proteins and allows 1,000-10,000 fold purification in one step. These techniques are well documented in Chapter 13 of Antibodies: A laboratory manual. by Harlow and Lane, Cold Spring Harbor Laboratories, (1989), incorporated herein by reference. The process involves utilizing antibody or antibody material to bind the reactive antigen to a solid substrate, washing away contaminating proteins, and then selectively removing the antigen. Variations of many formats can be utilized to accomplish this; one such example is as follows.

Neutrophils are isolated as described herein, then solubilized on ice with 1% NP-40 in phosphate-buffered saline. The lysate is centrifuged at 300 X g, and the

pelleted nuclear fraction (containing the UCpANCA antigen) is collected. The 5-3 recombinant UCpANCA Fab clone is incubated on ice with the nuclear fraction to permit formation of an 5-3 UCpANCA Fab immune complex with antigen. The antigen-antibody complexes are solubilized by DNAse I digestion, and the soluble complexes are isolated by affinity chromatography with Ni-NTA (Hochuli, E., Piesecki, S. (1992): Interaction of hexahistidine fusion proteins with nitrolotriacetic acid-chelated Ni2+ ions. Methods 4:68-72, incorporated herein by reference.) Molecular weight of the isolated antigen is assessed by SDS-PAGE. Based on this information, the protocol is up-scaled for preparative antigen solution.

Microsequencing of the isolated protein is used to design degenerate oligonucleotide primers, and these are employed in PCR cloning of the gene from a bone marrow or neutrophil cell line cDNA library (Goldsborough, A., Ashworth, A., Willison, K., Nucleic Acids Res 18:1634 (1990), incorporated herein by reference. Alternatively, cloning of the UCpANCA antigen is performed by direct screening of an expression cDNA library derived from bone marrow or neutrophil cell line cDNA. See, Aruffo, A., Seed, B., Proc Natl Acad Sci USA 84:8573-8577 (1987), incorporated herein by reference. These expression clones provide a source of UCpANCA antigen in sufficient quantities for production of commercially useful amounts of UCpANCA antigen for diagnostic and other commercial purposes.

The invention will now be described in greater detail by reference to the following non-limiting examples.

EXAMPLES

Example 1 - Isolation of PBL

Peripheral blood lymphocytes (PBL) were isolated directly by Ficoll-Hypaque fraction from 17 UC patients for analysis of pANCA production. All 17 of these UC patients were seropositive for pANCA by neutrophil ELISA, 16 of which demonstrated a pANCA staining pattern and the other displayed a cANCA staining pattern by the fixed neutrophil indirect immunofluorescence assay (IIF assay).

More specifically, 31.8 g Ficoll 400 (Pharmacia, Sweden) is combined with 400 ml deionized H_2O, shaken vigorously until dissolved and 100 ml of 50% sodium diatrizoate hypaque (UCLA Pharmacy, Los Angeles, California) is added and mixed. Specific gravity is checked using a hydrometer. It should be 1.077-1.080, preferably, 1.080. The Ficoll-hypaque solution is then filter-sterilize through a 0.22 or 0.45 μm bottle top filter. The Ficoll-hypaque solution may be stored at 4°C, protected from light.

Ficoll-hypaque solution (15 ml) is poured into 50 ml conical centrifuge tube, carefully overlaid with 30 ml heparinized blood and centrifuge at 1000 x g (2000 RPM) for 20 minutes. Using a serologic pipet or Pasteur pipet the interface is removed, placed into 50 ml conical centrifuge tube and dilute with at least an equal volume of Hanks' Balanced Salt Solution (HBSS) (Irvine Scientific, Santa Ana, California). The diluted interface is centrifuge at 400 x g (1200 RPM) for 5 minutes and the supernatant decanted. The pellet is re-suspended in 50 ml HBSS. Centrifugation and re-suspension of pellet is repeated twice and the PBL re-suspended in RPMI 1640 (Irvine Scientific, Santa Ana, California) + 5% fetal calf serum (GIBCO, Gathersberg, Maryland).

Example 2 - Isolation of LPL

Lymphocytes were isolated from biopsy tissue of an inflamed region of the lamina propria of a UC patient having high titer serum pANCA. Biopsy tissue is minced and then incubated at 37°C for 30 minutes in sterile culture medium (RPMI 1640, 10% FCS, and antibiotics) with 20 μg/ml collagenase, 20 μg/ml hyaluronidase, and 0.1% DNase. Tissue is then titrated through an 18 gauge needle until a cloudy suspension is achieved. After washing, the resultant single cell suspension is centrifuged on a Ficoll-Hypaque gradient to obtain mononuclear cells.

For surgical resections, the tissue is washed in saline to remove adherent debris, and the mucosa is dissected away from underlying layers. To remove the epithelial layer, small sections of mucosa (1 X 3 cm) are shaker-bath incubated in Ca^{2+}/Mg^{2+}-free Hank's Balanced Salt Solution ("HBSS") with 1 mM EDTA and antibiotics through serial medium changes. The remaining tissue is minced and digested in HBSS with 0.5 mg/ml collagenase and 1 mg/ml hyaluronidase, then processed as for biopsy specimens.

Isolated lymphocytes were cultured at 37°C in a humidified atmosphere of 5% CO_2:95% air for 12 days at a concentration of 2 x 10^6 cells/ml in RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal bovine serum and antibiotics.

Example 3 - Isolation of Neutrophil

Neutrophils were isolated from peripheral blood of normal individuals by Ficoll-Hypaque density centrifugation (specific gravity 1.080) followed by dextran sedimentation.

Accordingly, Ficoll-hypaque solution (15 ml) prepared as described in EXAMPLE 1 is poured into 50 ml conical centrifuge tube, carefully overlaid with 30 ml heparinized blood and centrifuge at 1000 x g (2000 RPM) for 20 minutes. The supernatant is carefully removed from the red blood cell pellet, 10 ml 6% dextran is added to 15 milliliters of pellet and topped off with 1X HBSS to 50 ml. The pellet is re-suspended and then the red blood cells allowed to settle, approximately 45 minutes to one hour. Supernatant is separated, topped off with 1X HBSS to 50 ml and centrifuged for 5 minutes at 1800 rpm. The supernatant is decanted and the pellet tapped. Remaining red blood cells are hypotonically lysed by adding 9 ml deionized water, swirling, then adding 1 ml 10X HBSS and immediately diluting with 1X HBSS to 50 ml. The lysed cells are centrifuged for 5 minutes at 1000 rpm. The supernatant is discarded and the neutrophil pellet re-suspended in 15 ml 1X HBSS.

Example 4 - Immobilization and Alcohol Fixation of Neutrophil on Microtiter Plate

Isolated neutrophil are re-suspend in sufficient volume of 1X HBSS to achieve 2.5 x 10^6 cells per ml. 0.1 ml of the cell suspension is added to each well of a 96-well microtiter Immulon 1™ or Immulon™ plate (available from Dynatech Laboratories of Chantilly, Virginia) and the cells allowed to settle for 30-60 minutes. The supernatant is removed with 8 channel manifold connected to a vacuum and plates are air dried (approximately 2 hours) or turned upside down on the grate of a laminar flow hood to dry (approximately 10 minutes).

Neutrophil are fixed with alcohol by incubating cells for 10 minutes in 0.1 ml of 100% methanol per well. The methanol is then discarded and the plates air dried. Store

at -20°C. All neutrophil plates were used within 2 weeks of preparation.

Example 5 - Neutrophil ELISA

The following "neutrophil ELISA" was performed on several samples described herein to detect or confirm the presence of ANCA or antibody material having specificity for neutrophil.

Wells were blocked with 0.25% BSA in phosphate-buffered saline ("PBS") for one hour. Supernatants from lymphocytes, transformed cells, serum, or other test sample was diluted as described herein with PBS + 0.5% Tween 20 was added to the wells prepared in accordance with EXAMPLE 4, incubated for one hour and then washed five times with 0.5% Tween-PBS. Plates were then developed by adding alkaline-phosphatase-conjugated goat $F(ab')_2$ anti-human IgG specific antibody (Pierce) diluted 1/1000 in PBS-0.5% Tween to each well and allowing it to incubate for one hour. Wells were washed five times with 0.5% Tween-PBS and then three times with Tris-NaCl (50mM Tris, /50mM NaCl, pH 7.5). Immune complex was detected with p-nitrophenyl phosphate (one tablet, Sigma 104 pnPP per 5 ml 10% diethanolamine, 1 mM $MgCl_2$, pH 9.8.). Absorbances were measured at 405 nm using a Biorad or Particle Data ELISA reader.

Example 6 - Neutrophil Antigen ELISA

An ELISA format was also used to determine whether the antigenic source for binding of antibodies and antibody materials to fixed neutrophil was the result of the presence of one of the following cytoplasmic constituents of neutrophils: elastase, cathepsin G, myeloperoxidase, and

lactoferrin. These assays are referred to collectively as the "neutrophil antigen ELISA."

These assays, were performed in accordance with the same protocol described in EXAMPLE 5 above, except that the wells of microtiter plates were coated overnight at 4°C with elastase, cathepsin G, myeloperoxidase, or lactoferrin. Rabbit anti-antigen antibodies were run with each antigen as positive controls.

Example 7 - Kappa-Capture ELISA

The presence of soluble dimeric antibody material of UC+, and UCpANCA material of the kappa isotype was detected and quantified in the supernatant using an ELISA referred to herein as "kappa-capture ELISA".

This ELISA was performed by coating the wells of a microtiter plate with a 1/1000 dilution of unlabeled goat anti-human kappa IgG (Southern Biotechnology Associates) by incubating the antibody-containing plates overnight at 4°C. The remaining steps were performed at room temperature.

Wells were blocked with 0.25% BSA in phosphate-buffered saline ("PBS") for one hour. Supernatants diluted in PBS + 0.5% Tween 20 were added to the wells, incubated for one hour and then washed five times with 0.5% Tween-PBS. Plates were then developed by adding alkaline-phosphatase-conjugated goat $F(ab')_2$ anti-human IgG specific antibody (Pierce) diluted 1/1000 in PBS-0.5% Tween to each well and allowing it to incubate for one hour. Wells were washed five times with 0.5% Tween-PBS and then three times with Tris-NaCl (50mM Tris, /50mM NaCl, pH 7.5). Immune complex was detected with p-nitrophenyl phosphate (one tablet, Sigma 104 pnPP per 5 ml 10% diethanolamine, 1 mM $MgCl_2$, pH 9.8.). Absorbances were measured at 405 nm using a Biorad or Particle Data ELISA reader.

Example 8 - Immobilization and Alcohol Fixation of Neutrophil on Glass Slides

Isolated neutrophil are re-suspend in sufficient volume of 1X HBSS to achieve 2.5 x 10^6 cells per ml. Neutrophil in suspension (0.1ml) are placed on a glass slide and the cells applied to the slide by using a cytospin at 500 rpm for 5 minutes.

The immobilized neutrophil are fixed by incubating slides for 10 minutes in sufficient volume of 100% methanol to cover sample. The slides are allowed to air dry and can be stored at -20°C.

Example 9 - Alcohol Fixed Indirect Immunofluorescence Assay

To slides prepared in accordance with EXAMPLE 8 above, 0.05 ml of serum diluted 1/40 in phosphate buffered saline, UCpANCA material or heterodimeric antibody material of UC+ diluted 1/500 in phosphate buffered saline, or undiluted supernatant from cultured PBL, MNL or LPL is added and allowed to incubate for one hour at room temperature. 0.05 ml phosphate buffered saline is added to clean slides as blanks

After three washes with PBS-Tween, slides were stained by adding 0.05 ml FITC-labeled goat F(ab')2 anti-human IgG antibody material (Jackson Immuno-Research, Westgrove, PA) at a 1:1000 antibody:phosphate buffered saline dilution and allowing to incubate for one hour at room temperature. After washing with about 100-250 ml phosphate buffered saline per slide to remove unbound anti-IgG antibody, the slides were allowed to air dry and were viewed on a fluorescein microscope equipped with epifluorescent optics. A pANCA staining pattern indicates the presence of UCpANCA.

Example 10 - DNase Sensitivity Assay

The DNase sensitivity assay was performed in an IIF assay format. Accordingly, the same protocol as described in EXAMPLE 9, above, was followed. Then the protocol was repeated for a second sample derived from the same source, except that before adding the sample to the slides, the neutrophils were incubated with one unit per slide of DNase I, for example, DNASCI™ (Sigma), per milliliter buffer (40mM Tris-HCl, 10mM NaCl, 6mM $MgCl_2$, 10mM $CaCl_2$, pH 7.9) for 30 min at 37°C. The slides were then washed three times with PBS, the sample added and the remainder of the IIF assay protocol followed. The staining patterns of neutrophil with the first sample and the staining pattern of the DNase-treated neutrophil with the second sample were compared to detect disruption of ANCA-antigen binding. The presence of a pANCA staining pattern in the first sample and the absence of a pANCA staining pattern or the presence of a cANCA staining pattern in the second sample indicates the presence of UCpANCA material in the samples.

Example 11 - Confocal Microscopy

Neutrophil (100,000/slide) were settled onto glass slides for 30 min at room temperature, fixed with 10 milligram paraformaldehyde per milliliter PBS for 10 minutes at room temperature, and incubated with pure acetone at -20°C for 1 minute. Slides were then air dried and stored at -20°C. Additionally, the neutrophil cell nucleus was visualized by counterstaining with propidium iodide, a DNA specific dye. Sera from UC patients, the UC patient whose cells were used to prepare the antibody phage library, and UCpANCA Fab (at 1/20 dilution for sera and at 1/100 dilution for Fab) were incubated with fixed neutrophils for one hour

at room temperature. After three washes with PBS-Tween, immune complex was labeled by incubating neutrophils with 1/1000 FITC-labeled goat-$F(ab')_2$ anti-human IgG-specific antibody material for one hour at room temperature. After washing, confocal images were taken on a Bio-Rad UV MRC-1000 confocal laser scanning system with a 60x objective lens (N.A. 1.4) attached to a Nikon 200 Diaphot microscope equipped with epifluorescence optics. The confocal micrographs were made with a Mitsubishi S3600 dye sublimation digital color printer.

Example 12 - Immune Electron Microscopy

Neutrophils were prepared by rapid freezing, freeze-dried by molecular distillation, stabilized by paraformaldehyde vapors and infiltrated with LR Gold. Ultra-thin sections were reacted with either UC$^+$ serum (1:5 and 1:10 dilution) or normal control serum and the antigen-antibody reaction detected by binding of protein G-gold or protein A-gold conjugate (5 nm colloidal gold) diluted 1:100. Protein G-gold, protein A-gold and buffer controls were included. Antibody to histone was used as a positive control for nuclear staining. All sections were finally fixed in Trumps solution (1% glutaraldehyde/4% paraformaldehyde, pH 7.2) washed and air dried. Sections were carbon coated and observed at 22,000-35,000X magnification using a Philips CM 12 electron microscope operating at an accelerating voltage of 40 KV.

Example 13 - Determination of ANCA Isotype

Supernatant of cultured LPL was analyzed for concentrations of total serum IgG by a standard ELISA method. Briefly, wells of microtiter plates (Costar,

Pleasanton, CA) were coated overnight (4°C) with goat anti-human IgG (Southern Biotechnology Associates, Birmingham, AL) diluted in carbonate-bicarbonate buffer, pH 9.6 (Sigma, St. Louis, MO). The plates were rinsed three times for 15 minutes with PBS + 0.5% Tween-20 and incubated for 1 hour at 4°C with serial dilutions for each serum sample, assayed in triplicate, then stained for one hour at 4°C with goat anti-human IgG-horseradish peroxidase (Southern Biotechnology Associates). Samples were incubated with a o-phenylenediamine (OPD) dihydrochloride substrate (Sigma) for 30 minutes at 37°C, arrested with 3-N H_2SO_4, and the absorbance determined at 492 nm. An IgG standard binding curve was established using a human IgG standard purified from pooled human serum (Sigma). The sample values were interpolated from the standard curve using a Macintosh ELISA program (Biorad, Richmond, CA).

Example 14 - Library Construction

V_H- and V_L-encoding DNA homolog libraries of the heavy and light chain gene repertoire of LPL cells from humans diagnosed with UC and seropositive for pANCA were randomly combined, expressed and the resulting antibody material screened for ability to bind neutrophil using a phage display technique. These variable heavy and light chain libraries were constructed by PCR cloning of variable heavy and light chains from these LPL. The homologs from these libraries were randomly paired in the dicistronic phagemid expression vector pComb 3 as described herein, resulting in a variable heavy chain fusion protein containing the V_H polypeptide and a fragment of the filamentous phage coat protein III. *E. coli* were subsequently transformed with these vectors containing the DNA-encoding heterodimeric antibody material. Expression of the vectors was induced

and the cells transformed with helper phage. Phage that were extruded from the transformed *E. coli* encapsulated the vector DNA encoding the nucleotide sequence and displayed the encoded heavy and light chains as Fab antibody material anchored to the phage coat via the gene III anchor protein. This phagemid expression system thus links both the process of recognition and replication in a single phage particle.

In a process called panning as described by Parmley et al., Gene, 74: 305-318 (1988), the phage expressing heterodimeric antibody material having anti-neutrophil immunoreactivity are enriched and isolated. The heterodimeric antibody material is then assayed for further UCpANCA characterization and the nucleic acid encoding representative UCpANCA Fab are sequenced.

V_H and V_L Library Generation

Nucleotide sequences encoding immunoglobulin protein CDRs are highly variable. However, there are several regions of conserved sequences that flank the V domains of the light and heavy chains that contain substantially conserved nucleotide sequences, i.e., sequences that will hybridize to the same primer sequence.

Polynucleotide synthesis ("amplification") primers that hybridize to these conserved sequences and incorporate restriction sites into the DNA homolog produced, restriction sites that are suitable for operatively ligating the DNA homolog to a vector, were constructed. More specifically, the primers are designed so that the resulting DNA homologs produced can be inserted into an expression vector in reading frame with the upstream translatable DNA sequence at the region of the vector containing the directional ligation means. Amplification with the primers described herein is performed on cDNA templates produced from total RNA isolated

from LPL of a human diagnosed with UC and seropositive for pANCA.

V_H Primers

For amplification of the V_H domains, primers are designed to introduce cohesive termini compatible with directional ligation into the unique Xho I and Spe I sites of the Hc2 expression cassette of the pComb 3 phagemid expression vector. In all cases, the 5' primers listed in SEQ ID NOs: 10 through 16 are chosen to be complimentary to the first strand cDNA in the conserved N-terminus region (anti-sense strand).

Additional V_H amplification primers, including the unique 3' primer, are designed to be complimentary to a portion of the first constant region domain of gamma 1 heavy chain mRNA (SEQ ID NO: 9). These primers will produce DNA homologs containing polynucleotides coding for amino acids from the V_H domain and the first constant region domain of immunoglobulin heavy chains of the IgG isotype. These DNA homologs can therefore be used to produce Fab fragments rather than F_V.

Additional unique 3' primers designed to be hybridized to similar regions of another class of immunoglobulin heavy chain such as IgM, IgE and IgA are contemplated. Other 3' primers that hybridize to a specific region of a specific class of CH_1 constant region and are adapted for transferring the V_H domains amplified using this primer to an expression vector capable of expressing those V_H domains with a different class of heavy or light chain constant regions are also contemplated.

Amplification is performed in seven separate reactions, each containing one of the 5' primers shown in SEQ ID NOs: 10 through 16, and a 3' primer shown in SEQ ID NO: 9. The 5' primers incorporate a Xho I site and the 3' primers

incorporate a Spe I restriction site for the insertion of the V_H-encoding DNA homolog into the pComb 3 phagemid Hc2 expression cassette.

V_L Primers

For amplification of the V_L domains, amplification primers are constructed that hybridize to the conserved sequences of immunoglobulin light chains and that incorporate restriction sites that allow cloning the V_L-encoding DNA homologs into the pComb 3 phagemid Lc2 expression cassette cut with Sac I and Xba I.
The 5' primers (SEQ ID NOs: 18 through 20) are designed to be complimentary to the first strand cDNA in the conserved N-terminus region. These primers also introduce a Sac I restriction endonuclease site to allow the V_L-encoding DNA homologs to be cloned into the pComb 3 phagemid Lc2 expression cassette. The 3' V_L amplification primer (SEQ ID NO: 17) is designed to hybridize to the constant region of kappa cDNA and to introduce the Xba I restriction endonuclease site required to insert V_L-encoding DNA homologs into the pComb 3 phagemid Lc2 expression cassette. These primers allow DNA homologs to be produced that encode immunoglobulin light chains of the kappa isotype. These primers make it possible to produce a Fab fragment rather than a Fv.

Amplification of the immunoglobulin light chain gene repertoire is performed in three separate reactions, each containing one of the 5' primers (SEQ ID NOs: 18 through 20) and one of the 3' primers (SEQ ID NO: 17). The 5' primers contain a Sac I restriction site and the 3' primers contain the Xba I restriction site.

Amplification primers designed to amplify human light chain variable regions of the lambda isotype are also contemplated.

All primers and synthetic polynucleotides described herein, were purchased from Oligos etc. (Wilsonville, OR).

V_H and V_L Library Construction

Total RNA was extracted from 1.15 x 10^7 lymphocytes using standard guanadinium isothiocynate extraction protocols. See, for example, Chomcynski, P. and Saochi, N., Anal. Biochem. 162:156-159 (1987), incorporated herein by reference.

In preparation for PCR amplification, the RNA, prepared above, is used as a template for cDNA synthesis by a primer extension reaction. Thus, 10 μg RNA was reverse transcribed to single-stranded cDNA using 1 μg oligo-dT primer with 10 mM dithiothreitol, RNasin™ (a protein RNase inhibitor of Promega Corporation, Madison, WI), 25 mM each dATP, dCTP, dGTP, dTTP, 1x reverse transcriptase buffer (Bethesda Research Laboratories, Bethesda, MD), and 2μl (two hundred units) reverse transcriptase (Superscript, Bethesda Research Laboratories) in 50 μl volume for 10 minutes at room temperature followed by 50 minutes at 42°C. Following a 5 minute 90°C heat kill and 10 minutes on ice, the reaction was treated with 1 μl (one unit) RNase H (Bethesda Research Laboratories) for 20 minutes at 37°C.

The single-stranded cDNA generated above was amplified using the polymerase chain reaction ("PCR") method. Family specific variable region and isotype specific constant region primers as described below were used to create heavy chain IgG1 V_H1-V_H6 and kappa light chain V_L1-V_L3 specific libraries:

Primer to create IgG1 heavy chain constant region library:

CG1z 5' GCATGTACTAGTTTTGTCACAAGATTTGGG 3' (SEQ ID NO:9)

Primers to create heavy chain variable region library:

V_H1a	5' CAGGTGCAGCTCGAGCAGTCTGGG 3'	(SEQ ID NO:10)
V_H2f	5' CAGGTGCAGCTACTCGAGTCGGG 3'	(SEQ ID NO:11)
V_H3a	5' GAGGTGCAGCTCGAGGAGTCTGGG 3'	(SEQ ID NO:12)
V_H3f	5' GAGGTGCAGCTGCTCGAGTCTGGG 3'	(SEQ ID NO:13)
V_H4f	5' CAGGTGCAGCTGCTCGAGTCGGG 3'	(SEQ ID NO:14)
V_H6a	5' CAGGTACAGCTCGAGCAGTCAGG 3'	(SEQ ID NO:15)
V_H6f	5' CAGGTACAGCTGCTCGAGTCAGGTCCA 3'	(SEQ ID NO:16)

Primer to create Kappa light chain constant region library:

C_K1d 5' GCGCCGTCTAGAACTAACACTCTCCCCTGTTGAAGC TCTTTGTGACGGGCGATCTCAG 3' (SEQ ID NO:17)

Primer to create Kappa light chain variable region library:

V_K1a	5' GACATCGAGCTCACCCAGTCTCCA 3'	(SEQ ID NO:18)
V_K2a	5' GATATTGAGCTCACTCAGTCTCCA 3'	(SEQ ID NO:19)
V_K3a	5' GAAATTGAGCTCACGCAGTCTCCA 3'	(SEQ ID NO:20)

PCR amplification is performed in a 100μl reaction containing the products of the reverse transcription reaction (about 1 μl of 450 μl reaction of the single-stranded cDNA), 60 pm of 3'V_H primer (SEQ ID NO: 9), 60 pm of the 5' primer (one of SEQ ID NOs: 10 through 16), 8 μl of the mixture of dNTP's at 25 mM each, 10 μl of 10 x PCR Buffer (Perkin-Elmer), and 5 units of Tag DNA polymerase (Perkin-Elmer, Norwalk, CT). The reaction mixture is subjected to 30 cycles of amplification using a Perkin-Elmer 9600 thermocycler. Each amplification cycle included denaturing of cDNA at 94°C for 15 seconds, followed by annealing of primers at 52°C for 50 seconds, and amplification at 72°C for 90 seconds. This was followed by a 10 minute extension at 72°C. Efficient and reproducible DNA homolog synthesis was achieved with the primers defined herein, producing amplified cDNA V_H-encoding homologs having

a major band of about 680 bp and amplified cDNA V_K-coding homologs having a major band at about 660 bp.

After verifying by agarose gel electrophoresis that all amplifications were successful and that similar yields were achieved, the V_H-encoding and V_L-encoding DNA homologs were separately pooled and gel purified on 0.8% Seaplaque GTG Agarose (FMC, Rockland, ME) according to the manufacturer's directions.

Ligation of V_L-encoding DNA Homologs into Vector

Equal portions of the products from each light chain primer extension reaction were mixed to generate a pooled V_L library of UC+. The pooled V_L library was double-digested with 70 units XbaI per microgram pooled V_L library and 35 units SacI per microgram pooled V_L library. (All restriction enzymes are available from Boehringer-Mannheim, Indianapolis, IN.) Digested products were again gel purified as described above, and the region of the gel containing DNA fragments of about 660 bp was excised, extracted from agarose and ethanol precipitated. The resulting V_L DNA homologs represent a repertoire of kappa light chain polypeptide genes having cohesive termini adapted for directional ligation to the pComb 3 phagemid Lc2 expression cassette.

The pComb 3 phagemid Lc2 expression cassette is prepared for inserting a light chain DNA homolog by admixing 30 μg of the phagemid to a solution containing 280 units of Xba I and 160 units of Sac I restriction endonucleases and a buffer recommended by the manufacturer. This solution was maintained at 37°C for 3 hours. The solution was precipitated with 2 ml glycogen, 1/10 volume 3M NaAc, 2.5 volume ethanol, at -20°C for 1 hour, then pelleted and washed with 70% ethanol. The pellet was re-suspended in water and gel purified on .8% 1 x TAE Seplaque 676. A 4 Kb

band was excised, phenol extracted, $LiCl_3$ treated and ethanol precipitated the same as PCR products.

The Lc2 expression cassette was then ready for ligation with the V_L-encoding DNA homologs prepared above. These V_L-encoding DNA homologs were then directly inserted into the Xba I and Sac I restriction digested Lc2 expression cassette by ligating 0.45 μg of V_L DNA homolog into 1.4 μg of digested pComb 3 (kindly provided by Dr. Carlos Barbas III of the Scripps Research Institute, La Jolla, California and described in Barbas et al., Proc. Natl. Acad. Sci. USA 88:7978-7982 (1991), incorporated herein by reference) using 10 units ligase in 200 μl volume ligase buffer stored overnight at 25°C, and then heat killed by maintaining at 65°C for 15 minutes (Boehringer-Mannheim). DNA was precipitated, washed with 70% ethanol, and re-suspended in 15 μl 10 mM $MgCl_2$.

Transformation of Host with Vector Containing V_L-Library

Escherichia coli XLI-Blue cells (Stratagene, La Jolla, CA) were transformed with re-suspended DNA by electroporation: 300 μl of stock made by concentrating 1 liter of *E. coli* OD_{600} = .8 down to 4 ml of cells were electroporated with 15 μl DNA (≈ 2 μg) (all of ligation mix). Transformed cells were selected for by plasmid antibiotic resistance by growth super broth containing 100 μg/ml carbenicillin. The library size was 8.6×10^7 transformants with 6% background re-ligation.

Antibiotic resistant colonies were amplified by growth in liquid cultures at 37°C in super broth ("SB") medium (30 g tryptone, 20 g yeast extract, and 10 g 3[N-Morpoholino] propane-sulfonic acid (Mops) per liter of water, adjusted to pH 7) supplemented with 10 μg/ml tetracycline, 20 μg/ml carbenicillin, 40 mM glucose and

10 mM $MgCl_2$. pComb 3 phagemids encoding a kappa V_L polypeptide ("Kappa - pComb 3 phagemid") were isolated using Qiagen-tips™, an anion-exchange resin of Qiagen, Chatsworth, CA following manufacturer instructions. Isolated Kappa-pComb 3 phagemids were double-digested with 10 units XhoI and 3 units SpeI per microgram Kappa-pComb 3 phagemid. Reaction mix was ethanol precipitated and 4.7 Kb double cut phagemid was gel purified on .8% Seaplaque TAE gel as before. The Kappa-pComb 3 phagemids were now ready for ligation with the heavy chain library.

Ligation of V_H-encoding DNA Homologs into Vector and Transformation of Host

Equal portions of the products form each heavy chain primer extension were mixed to generate a pooled V_H-encoding DNA homolog library. The pooled V_H library was prepared for ligation into the Hc2 expression cassette of the Kappa-pComb 3 phagemid by digestion with Xho I and Spe I nucleases. Accordingly, the pooled V_H library was double-digested with 70 units XhoI and 17 units SpeI per microgram pooled V_H library. Then, .40 μg digested heavy chain library was ligated with 1.4 μg digested Kappa-pComb 3 phagemid, described above, using 10 units ligase in 200 μl volume ligase buffer. The reaction was stopped by a heat kill at 65°C for 15 minutes. DNA was precipitated, the pellet re-suspended in 15 μl 10 mM $MgCl_2$ and used to electroporate *E. coli* XLI-Blue cells. Electroporated cells were grown in SB, supplemented as described above, except that glucose was not included. The library size was 4.9 x 10^7 with 14% background re-ligation after heavy chain cloning. Presence of both V_H-and V_L-encoding DNA homologs in the vector was verified by restriction analysis, seven out of seven clones contains both homologs.

Ten milliliter cultures of electroporated *E. coli* XLI-Blue cells were then transferred to SB supplemented with 50 μg/ml carbenicillin, 10 μg/ml tetracycline, and 10 mM $MgCl_2$ and incubated for another hour. Cultured cells were then infected with 10^{12} VCS-M13 helper phage (Stratagene, La Jolla, CA) to initiate the generation of copies of the sense strand of the phagemid DNA. After adding helper phage the mixture was added to 100 ml of SB supplemented with 50 μl/ml carbenicillin, 10 μl/ml tetracycline, and 10 mM $MgCl_2$. The admixture containing the helper phage was then maintained for an additional 2 hours at 37°C to allow for filamentous bacteriophage assembly wherein the expressed heterodimeric antibody material of UC^+ fused to cpIII bacteriophage anchor domain were incorporated into the surface of the bacteriophage particle. After 2 hours the mixture was spiked with 70 μg/ml kanamycin to select for helper phage infected *E. coli* and then allowed to grow overnight at 37°C, 300 rpm. The phage were precipitated by centrifugation resulting in a bacterial cell pellet and a supernatant containing phage, with the titer of colony-forming units ("CFU") determined by plating on LB plates with 100 μg/ml carbenicillin.

Example 15 - Panning

Each well of a 24-well microtiter plate was coated with methanol-fixed neutrophils by adding 10^6 neutrophils, allowing them to settle, air dry and then fixing with 100% methanol. Each well was blocked for one hour at 37°C with 3% bovine serum albumin ("BSA") in Tris-buffered saline ("TBS"). Blocking solution was removed and 5 x 10^{11} phage in 250 μl TBS was added and allowed to incubate for two hours at 37°C. After washing, acid elution, and neutralization, the number of phage eluted was monitored by CFU.

Eluted phage were amplified by reinfecting *E. coli* XLI-Blue and the panning/amplification cycle repeated five times until there was at least 100 fold enrichment. In this manner a library of phage enriched for UCpANCA material was generated. For enrichment quantitation, aliquots of the original library were re-panned in parallel with each cycle of enrichment to control for daily fluctuations in phage recovery. Enrichment was calculated by ratio of phage on vs. off and compared to the unenriched library run on the same day. Panning was also performed in a 96 well format with 10^{11} phage per well to compare formats.

Example 16 - Preparation of Soluble Recombinant Anti-Neutrophil Antibody Material of UC and Library Screening

Preparation of soluble heterodimeric antibody material, specifically Fab, was performed by isolating phagemid using Qiagen-tips™ in accordance with the manufacturer's instructions. (Qiagen, Chatsworth, CA) Isolated phagemid was then digested with 17 units SpeI and 50 units NheI per microgram of phagemid to remove cpIII gene segment. The phagemid DNA was then gel-purified and self-ligated by using 10 units ligase per 1 μg phagemid and maintaining the reaction mixture overnight at 25°C. The reaction was stopped by maintaining it at 65°C for 15 minutes. 200 ng gel purified fragment was self ligated in 20 μl volume and used to transform *E. coli* XLI-Blue by electroporation at 0°C in .2 cm gap curette at 2.5 kV, 25 μF and 200 R using 40 μl of *E. coli* stock and 1 μl of ligation mix. Single colonies were picked from an LB agar plates containing 100 μl/ml carbenicillin and grown in 10 ml SB supplemented with 10 μg/ml tetracycline, 50 μg/ml carbenicillin, and 20 mM $MgCl_2$ for six hours. Cultures were then induced by the addition of 1 mM isopropyl 6-D-thiogalactopyranoside ("IPTG") (United

States Biochemicals, Cleveland, OH) and grown overnight. The phage were isolated by centrifugation resulting in a bacterial cell pellet and a supernatant containing phage. The supernatant was removed and analyzed for Fab production by kappa-capture ELISA as described above, detecting with goat anti-human Fab-alkaline phosphatase (Pierce, Rockland, IL). Ten clones each from the enriched and unenriched libraries were selected for comparison. Six out of the ten clones from the unenriched library produced significant amounts of Fab as assayed by kappa-capture ELISA. In contrast, ten out of ten clones from the enriched library produced Fab, indicating that the enriched library had positively selected for Fab expression.

These clones were also analyzed for neutrophil binding by neutrophil ELISA. None of the ten clones from the unenriched library bound neutrophil, whereas all samples clones from the enriched library demonstrated avid neutrophil binding.

Diversity of heavy and light chain usage in Fabs from enriched and unenriched libraries were monitored by digesting 4 μg of phagemid encoding a single Fab with 20 units of BSTN1 (New England Biolabs, Beverly, MA) and analyzing fragments on a 3% agarose gel. Each of thirty clones from the unenriched library showed a distinct restriction pattern, whereas the clones from the enriched library displayed only two clonal patterns. Clones representative of these two patterns (5-3 and 5-4) were therefore directly analyzed by DNA sequencing, as described below.

Example 17 - Purification of Fab

Both enriched and unenriched libraries were transferred from pComb 3 to $C_2AP313H_6$, a pComb 3 derivative which fuses

six histidine to the carboxy terminus of the Fab after SpeI and NheI digestion to remove the cpIII anchor domain. ($C_3AP313H_6$ was a gift of Carlos Barbas III, Scripps Research Institute, La Jolla, California). Libraries were moved by removing the V_H- and V_L- encoding polynucleotides from the Hc2 and Lc2 expression cassettes of pComb 3 and sequentially ligating them into $C_3AP313H_6$. *E. coli* XLI-Blue cells were transformed with the new phagemid by electroporation. Individual colonies were isolated by LB agar selection supplemented with 100 μl/ml carbenicillin.

The 5-3 clone from the enriched library was chosen for large scale purification. A single colony was picked and allowed to grow overnight in 10 ml SB, supplemented with 10 μg/ml tetracycline, 50 μg/ml carbenicillin, 10 mM $MgCl_2$, and 40 mM glucose. The bacterial culture was pelleted by centrifugation to remove glucose and the cell pellet transferred into one liter of SB containing 50 μg/ml carbenicillin and 20 mM $MgCl_2$. The XL1-Blue cells were grown at 37°C shaking at 300 rpm until absorbance (OD_{600}) was between 0.6-0.8. The cell culture was then induced with 4 mM IPTG to express the heterodimeric antibody material and grown at 30°C overnight. The cell culture was centrifuged to pellet the XL1-Blue cells and the pellet re-suspended in 30 ml sonication buffer (50 mM $NaPO_4$, 300 mM $NaCl_2$, 0.01% NaN_3, pH 7.9). The re-suspended cells were sonicated eight times in 15 seconds bursts at 50% power (40 watts micro sonic disrupter, Tekmar, Cincinnati, OH).

The sonicate was centrifuged at 15,000 rpm in a Beckman JA-20 centrifuge for 40 min at 4°C and the supernatant serially filtered through a 0.45 and a 0.22 micron Nytex filter (Amicon, Beverly, MA). Sonicate was immediately loaded at 20 ml/hr on a 1 ml NTA-Ni column (Qiagen) and washed with sonication buffer, typically 40-50 ml, until absorbance (OD_{280}) was <0.01. The column was then washed

with 10 ml of 10 mM imidazole in sonication buffer to remove contaminants, followed with 10 ml each of 100 mM, 250 mM, and 500 mM imidazole collecting 1 ml fractions monitored by OD_{280}. Aliquots were analyzed by SDS-PAGE 12% denaturing and reducing gel to determine where Fab eluted. Due to the presence of imidazole, samples with loading dye were not boiled, but denatured instead at 37°C for 10 min before loading. Typically, the Fab elutes in first 3 fractions of the 100 mM imidazole wash.

One milliliter fractions that contain Fab were then pooled and dialyzed (6-8 kD cutoff membranes) using Amicon dialysis membranes against PBS to remove imidazole. Samples were concentrated and any free heavy or light chain removed using a Centricon 50™, centrifugation-dialysis membrane from Amicon Corporation, Beverly, MA.

Curiously, the calculated antibody level in the purified fraction differed with total protein (Bio-rad Protein Assay, Richmond, CA) versus ELISA (anti-Kappa) determination. Per 1 liter bacterial culture, Fab yield was ~1 mg by total protein assay, versus ~0.1 mg by immunoassay. Since use of the proteins in this study utilized ELISA immunoreactivity, Fab concentrations are reported using the ELISA method.

The 5-3 Fab was characterized using the assays described herein. Strong binding (approx. 0.1 micrograms/milliliter) to fixed neutrophil in an ELISA format. It is also notable that 5-3 UCpANCA Fab is avid compared to UC serum, since optimal binding occurred at 1% serum (or approx. 0.1 milligrams/milliliter total IgG). Estimating that approx. 1% hyperimmune serum is antigen-specific, then the level of native pANCA IgG is approx. 1 microgram/ml, or similar in range to binding by monovalent Fab.

In inflammatory disorders, ANCA-type marker antibodies are specific for certain defined neutrophil proteins. The 5-3 UCpANCA Fab was tested in the neutrophil antigen ELISA for reactivity with cathespin G, elastase, myeloperoxidase and lactoferrin. No binding was detected up to 500 nanograms/ml of 5-3 UCpANCA Fab.

The 5-3 UCpANCA Fab was also tested by alcohol-fixed neutrophil IIF assay for the pANCA staining pattern. Immunofluorescent detection of neutrophil staining by 5-3 UCpANCA Fab yielded the same pANCA staining pattern produced by conventional UC serum. When the immunoreactivity of 5-3 UCpANCA Fab was tested for DNase sensitivity, as with conventional pANCA seropositive UC serum, DNase I treatment of neutrophil caused the complete loss of detectable pANCA staining pattern. In addition, confocal microscopy demonstrated that 5-3 UCpANCA Fab binds antigen located inside the nuclear envelop, a characteristic found in pANCA seropositive UC serum.

Example 18 - Nucleic Acid Sequencing

Nucleic acid sequencing was carried out on double-stranded DNA of the 5-3 and 5-4 clones using 5' and 3' primers for the heavy and light chains (SEQ ID NOs: 21 and 22, and SEQ ID NOs. 23 and 24, respectively) and Sequenase 1.0 (United States Biochemicals). Homology searches and lineups were performed using Genebank.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: CEDARS-SINAI MEDICAL CENTER
(B) STREET: 8700 BEVERLY BLVD
(C) CITY: LOS ANGELES
(D) STATE: CALIFORNIA
(E) COUNTRY: US
(F) POSTAL CODE (ZIP): 90048
(G) TELEPHONE: 310-855-5284
(H) TELEFAX: 310-967-0101

(A) NAME: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA
(B) STREET: 300 LAKESIDE DRIVE, 21ST FLOOR
(C) CITY: OAKLAND
(D) STATE: CALIFORNIA
(E) COUNTRY: US
(F) POSTAL CODE (ZIP): 94612-3550
(G) TELEPHONE: 310-206-4401
(H) TELEFAX: 310-206-3619

(ii) TITLE OF INVENTION: ANTI-NEUTROPHIL CYTOPLASMIC ANTIBODY MATERIAL ASSOCIATED WITH ULCERATIVE COLITIS AND RELATED METHODS AND KITS

(iii) NUMBER OF SEQUENCES: 24

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 699 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(F) TISSUE TYPE: Gut-associated lymphoid

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       (G) CELL TYPE: Lymphocyte

(vii)  IMMEDIATE SOURCE:
       (B) CLONE: 5-3

 (ix)  FEATURE:
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       (B) LOCATION: 1..699
       (D) OTHER INFORMATION: /codon_start= 1
           /product= "Human Heavy Chain of IgG ANCA
           associated with UC"

 (ix)  FEATURE:
       (A) NAME/KEY: misc_RNA
       (B) LOCATION: 1..15
       (D) OTHER INFORMATION: /product= "N-Terminal Tag"

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       (D) OTHER INFORMATION: /label= CDR1
           /note= ""CDR1" refers to Complimentarity
           Determining Region 1"

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       (D) OTHER INFORMATION: /label= FR2
           /note= ""FR2" refers to Framework Region 2"

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       (D) OTHER INFORMATION: /label= CDR2
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       (B) LOCATION: 205..300
       (D) OTHER INFORMATION: /label= FR3
           /note= ""FR3" refers to Framework Region 3"

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       (B) LOCATION: 301..327
       (D) OTHER INFORMATION: /label= CDR3
           /note= ""CDR3" refers to Complimentarity
           Determining Region 3"
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(B) LOCATION: 328..360
(D) OTHER INFORMATION: /label= FR4
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(D) OTHER INFORMATION: /label= CH1
/note= ""CH1" refers to Constant Segment 1 of the Heavy Chain"

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(B) LOCATION: 652..678
(D) OTHER INFORMATION: /label= Hinge
/note= ""Hinge" refers to Partial Hinge Segment of the Heavy Chain"

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(A) NAME/KEY: misc_RNA
(B) LOCATION: 679..699
(D) OTHER INFORMATION: /label= Hex-HTAG
/note= ""Hex-HTAG" refers to Hexahistidine Tag"

(ix) FEATURE:
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(B) LOCATION: 16..651
(D) OTHER INFORMATION: /label= Fd
/note= ""Fd" refers to the Fd of the Heavy Chain"

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(B) LOCATION: 16..300
(D) OTHER INFORMATION: /label= VHSEGMENT
/note= ""VHSEGMENT" refers to Variable Segment of the Heavy Chain"

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(D) OTHER INFORMATION: /label= D
/note= ""D" refers to Diversity Segment"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 316..360
(D) OTHER INFORMATION: /label= JH
/note= ""JH" refers to Joining Segment of the Heavy Chain"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 16..360
(D) OTHER INFORMATION: /label= VHDOMAIN

/note= ""VHDOMAIN" refers to Variable Domain of the Heavy Chain"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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AAC TAT GGC ATG CAC TGG GTC CGG CAG GCT CCA GGC AAG GGG CTG GAG       144
Asn Tyr Gly Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu
         35                  40                  45

TGG GTG GCA GGT ATT TCC TCT GAT GGA AGA AAA AAA AAG TAT GTA GAC       192
Trp Val Ala Gly Ile Ser Ser Asp Gly Arg Lys Lys Lys Tyr Val Asp
     50                  55                  60

TCC GTG AAG GGC CGA TTC ACC ATC TCC AGA GAC AAG TCC AAG AAC ACG       240
Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Lys Ser Lys Asn Thr
 65                  70                  75                  80

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TAC TGT GCG AAA TTG TCC CGC GCG GGT GGT TTT GAC ATC TGG GGC CAA       336
Tyr Cys Ala Lys Leu Ser Arg Ala Gly Gly Phe Asp Ile Trp Gly Gln
            100                 105                 110

GGG ACA ATG GTC ACC GTC TCT TCA GCC TCC ACC AAG GGC CCA TCG GTC       384
Gly Thr Met Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val
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Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala
    130                 135                 140

CTG GGC TGC CTG GTC AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG TCG       480
Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser
145                 150                 155                 160

TGG AAC TCA GGC GCC CTG ACC AGC GGC GTG CAC ACC TTC CCG GCT GTC       528
Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
                165                 170                 175

CTA CAG TCC TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG ACC GTG CCC       576
Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro
            180                 185                 190

TCC AGC AGC TTG GGC ACC CAG ACC TAC ATC TGC AAC GTG AAT CAC AAG       624
Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys
```

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AAA ACT AGT CAC CAC CAC CAC CAC CAC                                  699
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225                 230
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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 233 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Asn Tyr Gly Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu
         35                  40                  45

Trp Val Ala Gly Ile Ser Ser Asp Gly Arg Lys Lys Lys Tyr Val Asp
     50                  55                  60

Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Lys Ser Lys Asn Thr
 65                  70                  75                  80

Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr
                 85                  90                  95

Tyr Cys Ala Lys Leu Ser Arg Ala Gly Gly Phe Asp Ile Trp Gly Gln
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    130                 135                 140

Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser
145                 150                 155                 160

Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
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Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro
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            180                 185                 190

Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys
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Lys Thr Ser His His His His His His
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(2) INFORMATION FOR SEQ ID NO:3:

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(A) LENGTH: 732 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(F) TISSUE TYPE: Gut-associated lymphoid
(G) CELL TYPE: Lymphocyte

(vii) IMMEDIATE SOURCE:
(B) CLONE: 5-4

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..732
(D) OTHER INFORMATION: /codon_start= 1
/product= "Human Heavy Chain of IgG ANCA
associated with UC"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..15
(D) OTHER INFORMATION: /product= "N-Terminal Tag"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 16..93
(D) OTHER INFORMATION: /label= FR1
/note= ""FR1" refers to Framework Region 1"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 94..108

(D) OTHER INFORMATION: /label= CDR1
/note= ""CDR1" refers to Complimentarity Determining Region 1"

(ix) FEATURE:
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(B) LOCATION: 109..150
(D) OTHER INFORMATION: /label= FR2
/note= ""FR2" refers to Framework Region 2"

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(A) NAME/KEY: misc_RNA
(B) LOCATION: 151..201
(D) OTHER INFORMATION: /label= CDR2
/note= ""CDR2" refers to Complimentarity Determining Region 2"

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(B) LOCATION: 202..297
(D) OTHER INFORMATION: /label= FR3
/note= ""FR3" refers to Framework Region 3"

(ix) FEATURE:
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(B) LOCATION: 298..360
(D) OTHER INFORMATION: /label= CDR3
/note= ""CDR3" refers to Complimentarity Determining Region 3"

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(B) LOCATION: 361..393
(D) OTHER INFORMATION: /label= FR4
/note= ""FR4" refers to Framework Region 4"

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(A) NAME/KEY: misc_RNA
(B) LOCATION: 394..684
(D) OTHER INFORMATION: /label= CH1
/note= ""CH1" refers to Constant Segment of the Heavy Chain"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 685..711
(D) OTHER INFORMATION: /label= Hinge
/note= ""Hinge" refers to Partial Hinge Segment of the Heavy Chain"

(ix) FEATURE:
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(B) LOCATION: 712..732
(D) OTHER INFORMATION: /label= Hex-HTag
/note= ""Hex-HTag" refers to Hexahistidine Tag"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 16..684
(D) OTHER INFORMATION: /label= Fd
/note= ""Fd" refers to the Fd of the Heavy Chain"

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(B) LOCATION: 16..297
(D) OTHER INFORMATION: /label= VHSEGMENT
/note= ""VHSEGMENT" refers to Variable Segment of the Heavy Chain"

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(B) LOCATION: 298..363
(D) OTHER INFORMATION: /label= D
/note= ""D" refers to Diversity Segment"

(ix) FEATURE:
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(B) LOCATION: 364..408
(D) OTHER INFORMATION: /label= JH
/note= ""JH" refers to Joining Segment of the Heavy Chain"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 16..408
(D) OTHER INFORMATION: /label= VHDOMAIN
/note= ""VHDOMAIN" refers to Variable Domain of the Heavy Chain"

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Ser Cys Ala Ala Ser Gly Phe Thr Phe Arg Asn Tyr Gly Met His Trp
            20                  25                  30

GTC CGG CAG GCT CCA GGC AAG GGG CTG GAG TGG GTG GCA GGT ATT TCC       144
Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ala Gly Ile Ser
        35                  40                  45

TCT GAT GGA AGA AAA AAA AAG TAT GTA GAC TCC GTG AAG GGC CGA TTC       192
Ser Asp Gly Arg Lys Lys Lys Tyr Val Asp Ser Val Lys Gly Arg Phe
    50                  55                  60

TTC ATC TCC AGA GAC AAT TCC AAG AAC ACC CTG TAT CTG CAA TTG AAC       240
Phe Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr Leu Gln Leu Asn
65                  70                  75                  80
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AGC CTG AGA GCT GAG GAC ACG GCT GTC TAT TAC TGT GCG AAA GAT GAG        288
Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala Lys Asp Glu
                 85                  90                  95

TTT AGT TCT ACC CGG AAG AAC TTC TTG ACT GGT CAA TCA AAG ACC TTT        336
Phe Ser Ser Thr Arg Lys Asn Phe Leu Thr Gly Gln Ser Lys Thr Phe
            100                 105                 110

GCG GCC TAC TAC GGT ATG GAC GTC TGG GGC CAA GGG ACC ACG GTC ACC        384
Ala Ala Tyr Tyr Gly Met Asp Val Trp Gly Gln Gly Thr Thr Val Thr
        115                 120                 125

GTC TCC TCA GCC TCC ACC AAG GGC CCA TCG GTC TTC CCC CTG GCA CCC        432
Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro
    130                 135                 140

TCC TCC AAG AGC ACC TCT GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC        480
Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val
145                 150                 155                 160

AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG TCG TGG AAC TCA GGC GCC        528
Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala
                165                 170                 175

CTG ACC AGC GGC GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC TCA GGA        576
Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly
            180                 185                 190

CTC TAC TCC CTC AGC AGC GTG GTG ACC GTG CCC TCC AGC AGC TTG GGC        624
Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly
        195                 200                 205

ACC CAG ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC AGC AAC ACC AAG        672
Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr Lys
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225                 230                 235                 240

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(2) INFORMATION FOR SEQ ID NO:4:

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(A) LENGTH: 244 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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        35                  40                  45

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    50                  55                  60

Phe Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr Leu Gln Leu Asn
65                  70                  75                  80

Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala Lys Asp Glu
                85                  90                  95

Phe Ser Ser Thr Arg Lys Asn Phe Leu Thr Gly Gln Ser Lys Thr Phe
            100                 105                 110

Ala Ala Tyr Tyr Gly Met Asp Val Trp Gly Gln Gly Thr Thr Val Thr
        115                 120                 125

Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro
    130                 135                 140

Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val
145                 150                 155                 160

Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala
                165                 170                 175

Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly
            180                 185                 190

Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly
        195                 200                 205

Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr Lys
    210                 215                 220

Val Asp Lys Lys Ala Glu Pro Lys Ser Cys Asp Lys Thr Ser His His
225                 230                 235                 240

His His His His
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 642 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(F) TISSUE TYPE: Gut-associated lymphoid
(G) CELL TYPE: Lymphocyte

(vii) IMMEDIATE SOURCE:
(B) CLONE: 5-3

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..642
(D) OTHER INFORMATION: /codon_start= 1
/product= "Kappa Light Chain of ANCA associated with Ulcerative Colitis"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..3
(D) OTHER INFORMATION: /label= N-TerminalTag

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 4..285
(D) OTHER INFORMATION: /label= VKSEGMENT
/note= ""VKSEGMENT" refers to Variable Segment of the Kappa Light Chain"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 286..324
(D) OTHER INFORMATION: /label= JK
/note= ""JK" refers to Joining Segment of the Kappa Light Chain"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 325..642
(D) OTHER INFORMATION: /label= CK
/note= ""CK" refers to Constant Segment of the Kappa Light Chain"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 4..66
(D) OTHER INFORMATION: /label= FR1
/note= ""FR1" refers to Framework Region 1"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 67..102

(D) OTHER INFORMATION: /label= CDR1
/note= ""CDR1" refers to Complimentarity Determining Region 1"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 103..147
(D) OTHER INFORMATION: /label= FR2
/note= ""FR2" refers to Framework Region 2"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 148..168
(D) OTHER INFORMATION: /label= CDR2
/note= ""CDR2" refers to Complimentarity Determining Region 2"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 169..264
(D) OTHER INFORMATION: /label= FR3
/note= ""FR3" refers to Framework Region 3"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 265..291
(D) OTHER INFORMATION: /label= CDR3
/note= ""CDR3" refers to Complimentarity Determining Region 3"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 292..324
(D) OTHER INFORMATION: /label= FR4
/note= ""FR4" refers to Framework Region 4"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
GCC GAG CTC ACG CAG TCT CCA GGC ACC CTG TCT TTG TTT CCA GGG GAA        48
Ala Glu Leu Thr Gln Ser Pro Gly Thr Leu Ser Leu Phe Pro Gly Glu
 1               5                  10                  15

AGA GCC ACT CTC TCC TGC AGG GCC AGT CAG AGA ATT AGC ACC AGT TTC        96
Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Arg Ile Ser Thr Ser Phe
            20                  25                  30

TTA GCC TGG TAC CAG CAG AAG CCT GGC CAG TCT CCC AGG CTC CTC ATC       144
Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Arg Leu Leu Ile
        35                  40                  45

TTT GAT GCA TCC ACC AGG GCC CCT GGC ATC CCT GAC AGG TTC AGT GCC       192
Phe Asp Ala Ser Thr Arg Ala Pro Gly Ile Pro Asp Arg Phe Ser Ala
    50                  55                  60

AGT TGG TCT GGG ACA GAC TTC ACT CTC ACC ATC AGC AGA CTG GAG CCT       240
```

```
Ser Trp Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Arg Leu Glu Pro
 65                  70                  75                  80

GAA GAT TTT GCA GTC TAT TAC TGT CAA CAT TAT GGT GGG TCT CCC TGG      288
Glu Asp Phe Ala Val Tyr Tyr Cys Gln His Tyr Gly Gly Ser Pro Trp
                 85                  90                  95

ACG TTC GGC CAA GGG ACC AAG GTG GAA ATC AAG CGA ACT GTG GCT GCA      336
Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala
            100                 105                 110

CCA TCT GTC TTC ATC TTC CCG CCA TCT GAT GAG CAG TTG AAA TCT GGA      384
Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly
        115                 120                 125

ACT GCC TCT GTT GTG TGC CTG CTG AAT AAC TTC TAT CCC AGA GAG GCC      432
Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala
    130                 135                 140

AAA GTA CAG TGG AAG GTG GAT AAC GCC CTC CAA TCG GGT AAC TCC CAG      480
Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln
145                 150                 155                 160

GAG AGT GTC ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC AGC CTC AGC      528
Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser
                165                 170                 175

AGC ACC CTG ACG CTG AGC AAA GCA GAC TAC GAG AAA CAC AAA GTC TAC      576
Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr
            180                 185                 190

GCC TGC GAA GTC ACC CAT CAG GGC CTG AGC TCG CCC GTC ACA AAG AGC      624
Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser
        195                 200                 205

TTC AAC AGG GGA GAG TGT                                             642
Phe Asn Arg Gly Glu Cys
    210
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 214 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Ala Glu Leu Thr Gln Ser Pro Gly Thr Leu Ser Leu Phe Pro Gly Glu
  1               5                  10                  15

Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Arg Ile Ser Thr Ser Phe
             20                  25                  30
```

```
Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Arg Leu Leu Ile
        35                  40                  45

Phe Asp Ala Ser Thr Arg Ala Pro Gly Ile Pro Asp Arg Phe Ser Ala
    50                  55                  60

Ser Trp Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Arg Leu Glu Pro
65                  70                  75                  80

Glu Asp Phe Ala Val Tyr Tyr Cys Gln His Tyr Gly Gly Ser Pro Trp
                85                  90                  95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala
            100                 105                 110

Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly
        115                 120                 125

Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala
    130                 135                 140

Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln
145                 150                 155                 160

Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser
                165                 170                 175

Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr
            180                 185                 190

Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser
        195                 200                 205

Phe Asn Arg Gly Glu Cys
    210
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 645 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(F) TISSUE TYPE: Gut-associated lymphoid
(G) CELL TYPE: Lymphocyte

(vii) IMMEDIATE SOURCE:

(B) CLONE: 5-4

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..645
(D) OTHER INFORMATION: /codon_start= 1
/product= "Kappa Light Chain of ANCA associated with Ulcerative Colitis"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..3
(D) OTHER INFORMATION: /label= N-TerminalTag

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 4..285
(D) OTHER INFORMATION: /label= VKSEGMENT
/note= ""VKSEGMENT" refers to Variable Segment of the Kappa Light Chain"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 286..327
(D) OTHER INFORMATION: /label= JK
/note= ""JK" refers to Joining Segment of the Kappa Light Chain"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 328..645
(D) OTHER INFORMATION: /label= CK
/note= ""CK" refers to Constant Segment of the Kappa Light Chain"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 4..66
(D) OTHER INFORMATION: /label= FR1
/note= ""FR1" refers to Framework Region 1"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 67..102
(D) OTHER INFORMATION: /label= CDR1
/note= ""CDR1" refers to Complimentarity Determining Region 1"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 103..147
(D) OTHER INFORMATION: /label= FR2
/note= ""FR2" refers to Framework Region 2"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA

(B) LOCATION: 148..168
(D) OTHER INFORMATION: /label= CDR2
/note= ""CDR2" refers to Complimentarity
Determining Region 2"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 169..264
(D) OTHER INFORMATION: /label= FR3
/note= ""FR3" refers to Framework Region 3"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 265..294
(D) OTHER INFORMATION: /label= CDR3
/note= ""CDR3" refers to Complimentarity
Determining Region 3"

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 295..327
(D) OTHER INFORMATION: /label= FR4
/note= ""FR4" refers to Framework Region 4"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
GCC GAG CTC ACG CAG TCT CCA GGC ACC CTG TCT TTG TCT CCA GGG GAA        48
Ala Glu Leu Thr Gln Ser Pro Gly Thr Leu Ser Leu Ser Pro Gly Glu
  1               5                  10                  15

AGA GCC ACC CTC TCC TGC AGG GCC AGT CAG GGT GTT AGC AGC GGC TCC        96
Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Gly Val Ser Ser Gly Ser
             20                  25                  30

TTA GCC TGG TAT CAG CAG AAA GCT GGC CAG GCT CCC AGG CTC CTC ATC       144
Leu Ala Trp Tyr Gln Gln Lys Ala Gly Gln Ala Pro Arg Leu Leu Ile
         35                  40                  45

TAT GGT GCA TCC AGG AGG GCC ACT GGC ATC CCA GAC AGG TTC ACT GGC       192
Tyr Gly Ala Ser Arg Arg Ala Thr Gly Ile Pro Asp Arg Phe Thr Gly
     50                  55                  60

AGT GGG TCT GGG ACA GAC TTC ACT CTC ACC ATC ACC AGA CTG GAG CCT       240
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Thr Arg Leu Glu Pro
 65                  70                  75                  80

GAA GAT TTT GCA GTG TAT TAC TGT CAG CAG TAT GGT AGC TCC CAG GGA       288
Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Tyr Gly Ser Ser Gln Gly
                 85                  90                  95

TTC ACT TTC GGC CCT GGG ACC AAA GTG GAT CTC AAA CGA ACT GTG GCT       336
Phe Thr Phe Gly Pro Gly Thr Lys Val Asp Leu Lys Arg Thr Val Ala
            100                 105                 110

GCA CCA TCT GTC TTC ATC TTC CCG CCA TCT GAT GAG CAG TTG AAA TCT       384
```

```
Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser
        115                 120                 125

GGA ACT GCC TCT GTT GTG TGC CTG CTG AAT AAC TTC TAT CCC AGA GAG      432
Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu
    130                 135                 140

GCC AAA GTA CAG TGG AAG GTG GAT AAC GCC CTC CAA TCG GGT AAC TCC      480
Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser
145                 150                 155                 160

CAG GAG AGT GTC ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC AGC CTC      528
Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu
                165                 170                 175

AGC AGC ACC CTG ACG CTG AGC AAA GCA GAC TAC GAG AAA CAC AAA GTC      576
Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val
            180                 185                 190

TAC GCC TGC GAA GTC ACC CAT CAG GGC CTG AGC TCG CCC GTC ACA AAG      624
Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys
        195                 200                 205

AGC TTC AAC AGG GGA GAG TGT                                         645
Ser Phe Asn Arg Gly Glu Cys
    210                 215
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 215 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Ala Glu Leu Thr Gln Ser Pro Gly Thr Leu Ser Leu Ser Pro Gly Glu
  1               5                  10                  15

Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Gly Val Ser Ser Gly Ser
             20                  25                  30

Leu Ala Trp Tyr Gln Gln Lys Ala Gly Gln Ala Pro Arg Leu Leu Ile
         35                  40                  45

Tyr Gly Ala Ser Arg Arg Ala Thr Gly Ile Pro Asp Arg Phe Thr Gly
     50                  55                  60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Thr Arg Leu Glu Pro
 65                  70                  75                  80

Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Tyr Gly Ser Ser Gln Gly
                 85                  90                  95
```

```
Phe Thr Phe Gly Pro Gly Thr Lys Val Asp Leu Lys Arg Thr Val Ala
            100                 105                 110

Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser
        115                 120                 125

Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu
    130                 135                 140

Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser
145                 150                 155                 160

Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu
                165                 170                 175

Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val
            180                 185                 190

Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys
        195                 200                 205

Ser Phe Asn Arg Gly Glu Cys
    210                 215
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..30
(D) OTHER INFORMATION: /label= CG1z
/note= ""CG1z" refers to the cDNA Primer for IgG1 Heavy Chain Constant Segments"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GCATGTACTA GTTTTGTCAC AAGATTTGGG 30

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..24
(D) OTHER INFORMATION: /label= VH1a
/note= ""VH1a" refers to the cDNA Primer for Variable Segments of the Heavy Chain that are Members of the VH1 Gene Family"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CAGGTGCAGC TCGAGCAGTC TGGG 24

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..24
(D) OTHER INFORMATION: /label= VH3a
/note= ""VH3a" refers to the cDNA Primer for Variable Segments of the Heavy Chain that are Members of the VH3 Gene Family"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GAGGTGCAGC TCGAGGAGTC TGGG 24

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..23
(D) OTHER INFORMATION: /label= VH2f
/note= ""VH2f" refers to the cDNA Primer for Variable Segments of the Heavy Chain that are Members of the VH2 Gene Family"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CAGGTGCAGC TACTCGAGTC GGG 23

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..24
(D) OTHER INFORMATION: /label= VH3f
/note= ""VH3f" refers to the cDNA Primer for Variable Segments of the Heavy Chain that are Members of the VH3 Gene Family"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GAGGTGCAGC TGCTCGAGTC TGGG 24

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..23
(D) OTHER INFORMATION: /label= VH4f
/note= ""VH4f" refers to the cDNA Primer for Variable Segments of the Heavy Chain that are Members of the VH4 Gene Family"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CAGGTGCAGC TGCTCGAGTC GGG 23

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..23
(D) OTHER INFORMATION: /label= VH6a
/note= ""VH6a" refers to the cDNA Primer for Variable Segments of the Heavy Chain that are Members of the VH6 Gene Family"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CAGGTACAGC TCGAGCAGTC AGG 23

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..27
(D) OTHER INFORMATION: /label= VH6f
/note= ""VH6f" refers to the cDNA Primer for Variable Segments of the Heavy Chain that are Members of the VH6 Gene Family"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CAGGTACAGC TGCTCGAGTC AGGTCCA 27

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 58 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..58
(D) OTHER INFORMATION: /label= CK1d
/note= ""CK1d" refers to the cDNA Primer for Kappa Light Chain Constant Segments"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GCGCCGTCTA GAATTAACAC TCTCCCCTGT TGAAGCTCTT TGTGACGGGC GAACTCAG 58

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..24
(D) OTHER INFORMATION: /label= VK1a
/note= ""VK1a" refers to the cDNA Primer for Variable Segments of the Kappa Light Chain that are Members of the VK1 Gene Family"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GACATCGAGC TCACCCAGTC TCCA 24

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..24
(D) OTHER INFORMATION: /label= VK2a
/note= ""VK2a" refers to the cDNA Primer for Variable Segments of the Kappa Light Chain that are Members of the VK2 Gene Family"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GATATTGAGC TCACTCAGTC TCCA 24

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..24
(D) OTHER INFORMATION: /label= VK3a
/note= ""VK3a" refers to the cDNA Primer for Variable Segments of the Kappa Light Chain that are Members of the VK3 Gene Family"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GAAATTGAGC TCACGCAGTC TCCA 24

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..22
(D) OTHER INFORMATION: /note= "5' Heavy Chain Sequencing Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GGCCGCAAAT TCTATTTCAA GG 22

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..18
(D) OTHER INFORMATION: /note= "3' Heavy Chain Sequencing Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CGCTGTGCCC CCAGAGGT 18

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..18
(D) OTHER INFORMATION: /note= "5' Light Chain Sequencing Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CTAAACTAGC TAGTCGCC 18

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_RNA
(B) LOCATION: 1..21
(D) OTHER INFORMATION: /note= "3' Light Chain Sequencing Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

ATAGAAGTTG TTCAGCAGGC A 21

We claim:

1. Isolated antibody material associated with ulcerative colitis and immunoreactivity with nuclear antigen in neutrophils, wherein said immunoreactivity is characterized by a perinuclear staining pattern generated in an alcohol-fixed neutrophil indirect immunofluorescence assay.

2. The antibody material of claim 1, wherein said immunoreactivity is further characterized as being disrupted by pre-treatment of alcohol-fixed neutrophil with DNase.

3. The antibody material of claim 1, wherein said immunoreactivity is further characterized as localized within the nuclear envelop of the neutrophil.

4. The antibody material of claim 1, wherein said antibody material is a Fab having a molecular weight of about 60,000 Daltons on 12% SDS-PAGE.

5. Antibody material comprising anti-neutrophil cytoplasmic antibody material associated with ulcerative colitis, wherein said antibody material is characterized as having immunoreactivity with antigen localized within the nuclear envelop of neutrophil, wherein said immunoreactivity is characterized by a perinuclear staining pattern by alcohol-fixed neutrophil indirect immunofluorescence assay, and wherein said immunoreactivity is further characterized as being disrupted by pre-treatment of said neutrophil with DNase.

6. A recombinant polypeptide comprising at least one variable segment of an immunoglobulin heavy chain ("V_H segment"),

wherein the V_H segment comprises framework regions, complementarity determining region I ("CDR_H I")and complementarity determining region II ("CDR_H II"), and

wherein the CDR_H I has substantially the same amino acid sequence as the amino acid sequence selected from the group consisting of residues 33 through 37 of SEQ ID NO: 2 and residues 32 through 36 of SEQ ID NO: 4.

7. The polypeptide of claim 6, wherein the CDR_H II has substantially the same amino acid sequence as the amino acid sequence selected from the group consisting of residues 52 through 68 of SEQ ID NO: 2 and residues 51 through 67 of SEQ ID NO: 4.

8. The polypeptide of claim 7, further comprising an immunoglobulin heavy chain joining segment ("J_H segment") and a diversity segment, wherein at least a portion of the J_H segment and a portion of the diversity segment define a complementarity determining region III ("CDR_H III"), and wherein the amino acid sequence of the CDR_H III is substantially the same as the amino acid sequence selected from the group consisting of residues 101 through 109 of SEQ ID NO: 2 and residues 100 through 120 of SEQ ID NO: 4.

9. A recombinant polypeptide comprising at least one variable segment of an immunoglobulin heavy chain ("V_H segment"),

wherein the V_H segment comprises framework regions, complementarity determining region I

("CDR_H I")and complementarity determining region II ("CDR_H II"), and

wherein the CDR_H II has substantially the same amino acid sequence as the amino acid sequence selected from the group consisting of residues 52 through 68 of SEQ ID NO: 2 and residues 51 through 67 of SEQ ID NO: 4.

10. The polypeptide of claim 9, further comprising an immunoglobulin heavy chain joining segment ("J_H segment") and a diversity segment, wherein at least a portion of the J_H segment and a portion of the diversity segment define a complementarity determining region III ("CDR_H III"), and wherein the amino acid sequence of the CDR_H III is substantially the same as the amino acid sequence selected from the group consisting of residues 101 through 109 of SEQ ID NO: 2 and residues 100 through 120 of SEQ ID NO: 4.

11. An isolated polypeptide comprising an immunoglobulin heavy chain variable domain having substantially the same amino acid sequence as the amino acid sequence selected from the group consisting of residues 16 through 217 of SEQ ID NO: 2 and residues 6 through 136 of SEQ ID NO. 4.

12. An isolated polynucleotide encoding the polypeptide of claim 11.

13. Antibody material comprising the polypeptide of claim 11, wherein the antibody material is selected from the group consisting of Fab, Fab', $F(ab')_2$ and an antibody.

14. The antibody material of claim 13, wherein the antibody material is Fab, wherein said Fab consists of a

immunoglobulin heavy chain Fd and an immunoglobulin light chain.

15. The antibody material of claim 14, wherein the immunoglobulin light chain comprises substantially the same amino acid sequence as an amino acid sequence selected from the group consisting of residues 2 through 107 of SEQ ID NO: 6 and residues 2 through 108 of SEQ ID NO: 8.

16. A polynucleotide encoding the a polypeptide having substantially the same amino acid sequence as the amino acid sequence selected from the group consisting of residues 2 through 107 of SEQ ID NO: 6 and residues 52 though 108 of SEQ ID NO: 8.

17. A method for producing a library of dicistronic phagemid expression vectors encoding heterodimeric antibody material of an immunoglobulin gene repertoire of UC^+, comprising:

(a) forming a first ligation admixture by combining in a ligation buffer

(i) a first library of the immunoglobulin gene repertoire of UC^-, said first library comprising a plurality of DNA homologs in the form of dsDNA, each DNA homolog of the library having cohesive termini adapted for directional ligation, wherein said library is selected from the group consisting of a V_H library of UC^+ and a V_L library of UC^+, and

(ii) a plurality of phagemid expression vectors in linear form, each having upstream and downstream first cohesive termini that are adapted for directionally receiving a DNA homolog of the first library of the immunoglobulin gene repertoire of UC^+ in a common reading frame, and wherein said first cohesive termini

are operatively linked to respective upstream and downstream translatable DNA sequences, which in turn are operatively linked to respective upstream and downstream DNA expression control sequences;

(b) subjecting the admixture to ligation conditions for a time period sufficient to operatively link DNA homologs of the first library of the immunoglobulin gene repertoire of UC* to the vectors and produce a plurality of circular phagemid expression vectors each having a first cistron for expressing the first library of the immunoglobulin gene repertoire of UC*, wherein the upstream translatable DNA sequence of the first cohesive termini encodes a prokaryotic secretion signal and the downstream translatable DNA sequence of the first cohesive termini encodes a filamentous phage coat protein membrane anchor;

(c) treating the plurality of circular phagemid expression vectors under DNA cleavage conditions to produce a plurality of phagemid expression vectors in linear form that each have upstream and downstream second cohesive termini that

(i) are adapted for directionally receiving a DNA homolog of a second library of the immunoglobulin gene repertoire of UC* in a common reading frame, and

(ii) are operatively linked to respective upstream and downstream translatable DNA sequences which in turn are operatively linked to DNA expression control sequences, wherein the upstream DNA sequence of the second cohesive termini is a translatable sequence encoding a prokaryotic secretion signal and the downstream DNA sequence of the second cohesive termini has at least one stop codon in the reading frame;

(d) forming a second ligation admixture by combining in a ligation buffer

(i) the plurality of phagemid expression vectors formed in (c), and

(ii) the second library of the immunoglobulin gene repertoire of UC+, said second library comprising a plurality of DNA homologs in the form of dsDNA, each DNA homolog of the library having cohesive termini adapted for directional ligation to the second cohesive termini of the phagemid expression vectors, wherein said library is selected from the group consisting of a V_H library of UC+ and a V_L library of UC+; and

(e) subjecting the second admixture to ligation conditions for a time period sufficient to operatively link DNA homologs of the second library of the immunoglobulin gene repertoire of UC+ to said vectors, producing a plurality of circular phagemid expression vectors each having a second cistron for expressing the second library of the immunoglobulin gene repertoire of UC+.

18. A library of phagemid expression vectors containing cDNA homologs encoding V_L and V_H polypeptides from an immunoglobulin gene repertoire of UC+, wherein said immunoglobulin gene repertoire of UC+ is derived from LPL of a human diagnosed with UC and seropositive for pANCA.

19. A plurality of prokaryotic cells, containing the library of phagemid expression vectors of claim 18.

20. A population of filamentous phage particles encapsulating the library of claim 18.

21. The library of phagemid expression vectors contained in the population of *E. coli* deposited with the American Type Culture Collection and having ATCC Accession No. 69827.

22. A method for producing filamentous phage particles having on the particle surface heterodimeric antibody material of UC* comprising:

(a) introducing into a prokaryotic host cell permissive for filamentous phage replication one or more phagemid expression vectors containing and capable of expressing V_H-encoding and V_L-encoding DNA homologs of UC*, wherein one of the encoded polypeptides is fused to a filamentous phage coat protein membrane anchor and wherein both of the encoded polypeptides are each fused to a prokaryotic secretion signal, and

(b) maintaining the prokaryotic host cell containing the vector under conditions sufficient for filamentous phage production and under conditions sufficient for expression of the heterodimeric antibody material of UC*, thereby forming the phage particle.

23. A population of filamentous phage particles encapsulating dicistronic phagemid expression vectors encoding heterodimeric antibody material of UC*, wherein said heterodimeric antibody material immunoreacts with UCpANCA antigen as demonstrated by binding to alcohol-fixed neutrophil.

24. A method of detecting UCpANCA in a sample, comprising:

(a) contacting the sample and a detectable secondary reagent with fixed neutrophil under conditions suitable to form an immune complex of neutrophil, UCpANCA and detectable secondary reagent, wherein said secondary reagent has binding specificity for UCpANCA or the class determining portion of UCpANCA;

(b) separating unbound secondary reagent from immune complex; and

(c) assaying for the presence or absence of UCpANCA containing immune complex within the nuclear envelop of the neutrophil by detecting the presence or absence of bound secondary reagent.

25. A kit comprising UCpANCA material in an amount sufficient for at least one assay and instructions for use of UCpANCA material as a reference reagent in an immunoassay to screen for UC.

26. A method of isolating UCpANCA immunoreactive antigen comprising:

(a) contacting UCpANCA material with neutrophil cell lysate for a time, at a temperature and at a pH suitable to form an immune complex comprising UCpANCA material,

(b) separating said immune complex from non-complexed cell lysate,

(c) separating said UCpANCA material from said antigen.

27. Nucleic acid encoding UCpANCA antibody material.

28. An anti-idiotypic antibody having binding specificity for UCpANCA.

FIG. 1A

FIG. 1B

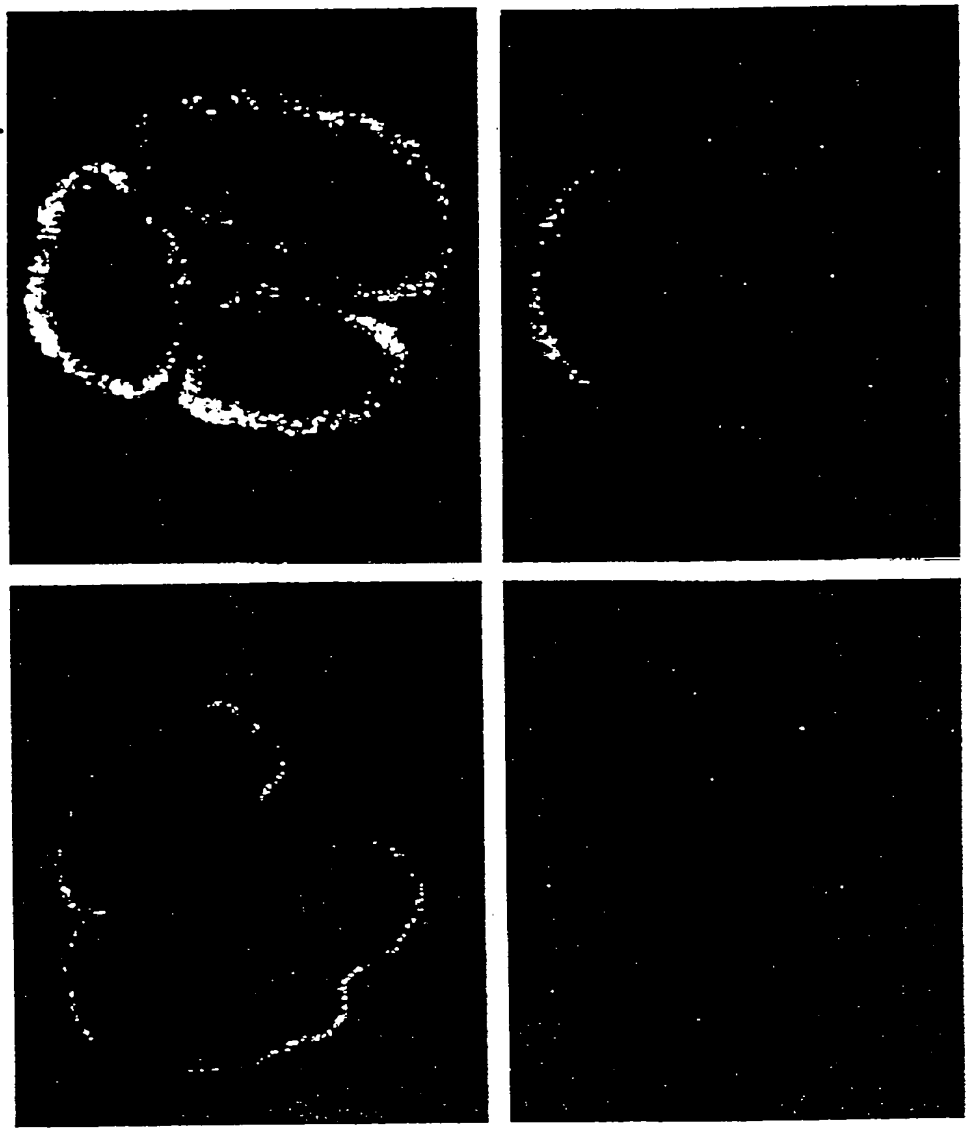
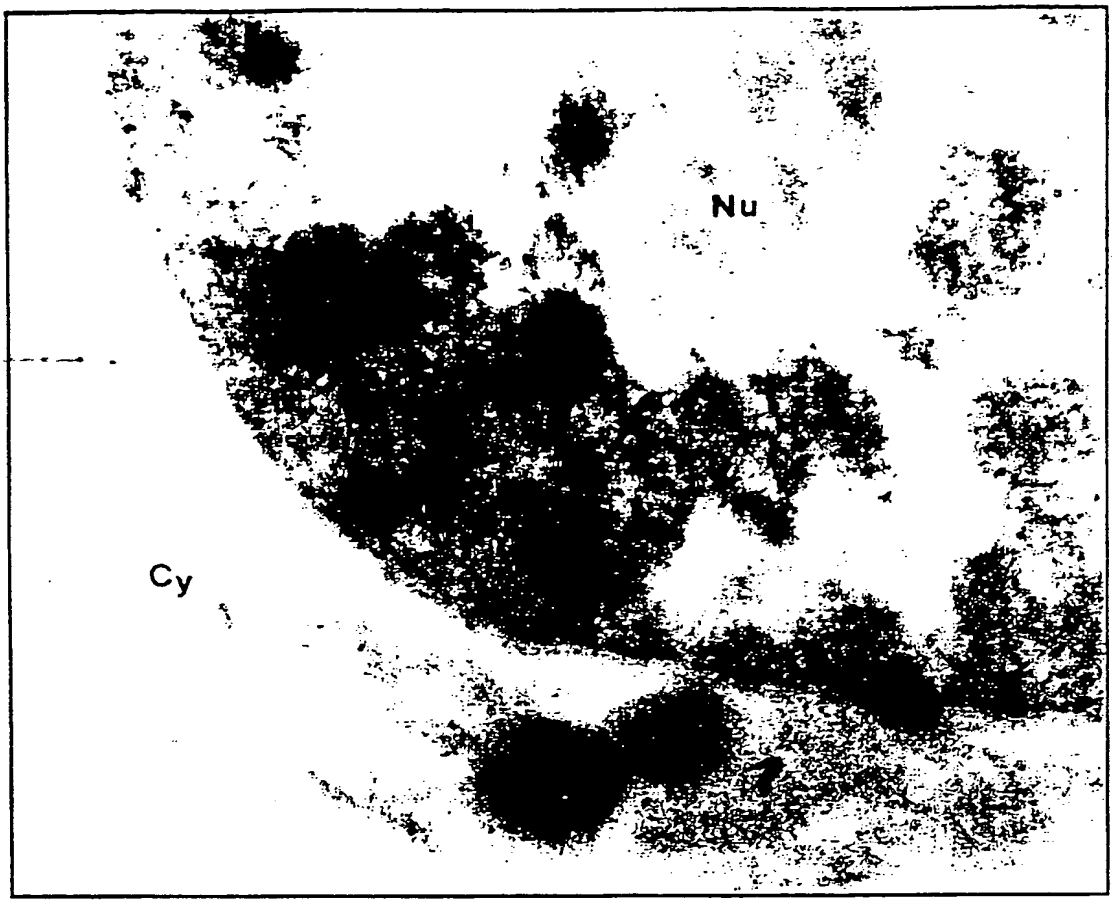


FIG. 1C

FIG. 1D

FIG. 2A



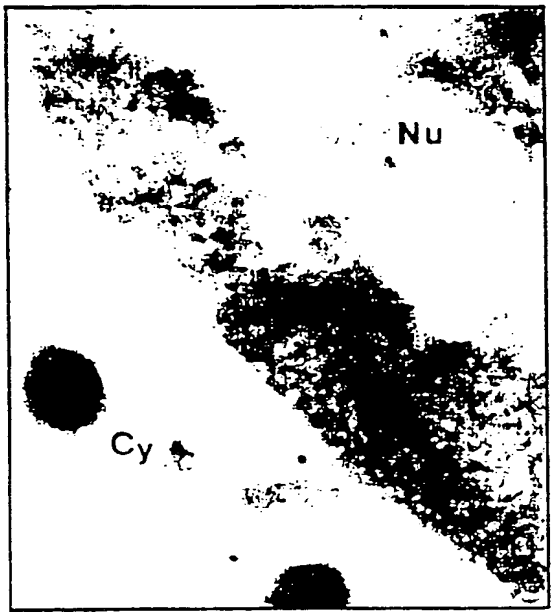


FIG. 2B

FIG. 2C

FIG. 3

(a) VARIABLE HEAVY CHAIN

	FR1	CDR1	FR2	CDR2
DP 49	LVESGGGVVQPGRSLRLSCAASGFTFS	SYGMH	WVRQAPGKGLEWVA	VISYDGSNKYYADSVKG
5-3	-EQ---------K----------R	N----	----------	G--S--RK-K-V-----
5-4	-E ---------K----------R	N----	----------	G--S--RK-K-V-----

	FR3	CDR3	FR4
DP 49	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK		
5-3	----------K----------	LSRAGGFDI	WGQGTMVTVSS
5-4	--F----------L----------	DEFSSTRKNFLTGQSKTFAAYYGMDV	WGQGTTVTVSS

(b) VARIABLE LIGHT CHAIN

	FR1	CDR1	FR2	CDR2-	FR3
VA27	VLTQSPGTLSLSPGERATLSC	RASQSVSSSYLA	WYQQKPGQAPRLLIY	GASSRAT	GIPDR
5-3	E----------F---------	----RI-T-F--	--------S-----F	D--T--P	-----
5-4	E----------	----G---GS--	-----A---------	---R---	-----

	FR3	CDR3	FR4
VA27	FSGSGSGTDFTLTISRLEPEDFAVYYC	QQYGSSP	
5-3	--A-W----------	-H----- WT	FGQGTKVEIKR
5-4	-T----------T----------	------QGFT	FGPGTKVDLKR

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/08756

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 39/395; C12N 15/70; C12Q 1/70; G01N 33/53; C07H 21/00
US CL :530/387.1, 387.2; 536/23.53; 435/7.93, 69.7, 235.1, 240.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/387.1, 387.2; 536/23.53; 435/7.93, 69.7, 235.1, 240.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS; Dialog
search terms: antibody, ulcerative colitis, inventors

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BERBERIAN, L.S. Expression of a novel autoantibody defined by the V sub(H)3-15 gene in inflammatory bowel disease and Campylobacter jejuni enterocolitis. J. Immunology. 1994, Vol. 153, No. 8, pages 3756-3763. See entire reference.	1-28
Y	VALLESAYOUB, Y. Characterization of a common VH3-15 autoantibody relating inflammatory bowel-disease and c-jejuni enterocolitis. FASEB J. 18 March 1994, Vol. 8, No. 5, page A1010, abstract number 5851. See entire document.	1-28

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 15 AUGUST 1996	Date of mailing of the international search report 11 SEP 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Donald E. Adams, Ph.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/08756

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EGGENA, M. Characterization of ulcerative colitis specific pANCA using phage display technology. FASEB J. 18 March 1994, Vol. 8, No. 5, page A1010, abstract number 5850. See entire abstract.	1-28